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TRIFLUOROACETOLYSIS

MODEL COMPOUND STUDIES AND APPLICATION IN
THE STRUCTURAL ANALYSIS OF THE CARBOHYDRATE
PORTION OF HUMAN ANTITHROMBIN III



Lund 1979



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MODEL COMPOUND STUDIES AND APPLICATION IN THE STRUCTURAL ANALYSIS
OF THE CARBOHYDRATE PORTION OF HUMAN ANTITHROMBIN III

by

Lars-Erik Franzén



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This thesis is a summary of the following papers:

- I The stability of partially methylated methyl α -D-glucopyranosides towards trifluoroacetolysis
L.-E. Franzén and S. Svensson
Carbohyd. Res. 73 (1979) 309
- II The stability of partially methylated methyl α -D-xylopyranosides and partially methylated D-xyloses towards trifluoroacetolysis
L.-E. Franzén and S. Svensson
Carbohyd. Res., in press
- III The stability of partially methylated methyl α -L-arabinofuranosides towards trifluoroacetolysis
L.-E. Franzén and S. Svensson
Acta Chem. Scand., in press
- IV The effect of hydroxyl groups in the aglycone in the solvolysis of glucopyranosides by trifluoroacetolysis
L.-E. Franzén and S. Svensson
Acta Chem. Scand., in press
- V Structural studies on the carbohydrate portion of human antithrombin III
L.-E. Franzén, S. Svensson and O. Larm
Submitted for publication



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1. INTRODUCTION

The term, glycoconjugate, is the common designation for glycoproteins, proteoglycans and glycolipids. In glycoproteins, the carbohydrate chains are linked N-glycosidically to asparagine and O-glycosidically to serine, threonine, hydroxylysine or hydroxyproline (1,2). The carbohydrate chains of proteoglycans have similar linkages to the protein part (3). The glycolipids are O-glycosidically linked and exist as glycosyldiglycerides in plants and bacteria and as glycosylceramides in humans and animals. Glycolipids based on dihydrosphingosine are also known from plants. A prerequisite for the elucidation of the structure of the carbohydrate moieties of glycoconjugates is that there exists methods for isolating the carbohydrate portions preferably without any degradation. If limited degradation occurs it should be clearly defined and if possible give structural information. Digestion of glycoproteins and proteoglycans by proteolytic enzymes, if successful, results in glycopeptides containing one or more amino acids. Hydrazinolysis (4) or alkaline borohydride treatment (5) are used to release N-glycosidically linked carbohydrate chains, whereas carbohydrate chains O-glycosidically linked to serine or threonine are split off by mild alkaline borohydride treatment (6). Isolation of the carbohydrate portion in glycosylceramides is effected by oxidative cleavage of the sphingosine double bond followed by alkaline β -elimination from the aglycone residue (7).

Trifluoroacetolysis offers a new and versatile method for the structural investigation of glycoconjugates. Trifluoroacetolysis is performed with various mixtures of trifluoroacetic

acid (TFA) and trifluoroacetic anhydride (TFAA) at different temperatures. With this reagent sugar hydroxyls are rapidly converted into strongly electron-withdrawing O-trifluoroacetyl groups that exert a stabilizing influence on the glycosidic bonds which is due to the diminished electron-density at the ring oxygens and the glycosidic oxygens. Most reducing sugars (8) and glycosides (9) are stable under conditions that will effect transamidation. Thus the protein part of glycoproteins will be degraded leaving the carbohydrate moiety intact except for some degradation of 2-acetamido-2-deoxy sugars at the reducing end (8,10,11). The transamidation constitutes a convenient method for the N-deacetylation of glycosidically linked sugar residues (12) since N-detrifluoroacetylation is easily accomplished with sodium borohydride or methanolic ammonia. N-Glycosidically linked carbohydrate chains are cleaved from glycoproteins by transamidation (10,11), whereas carbohydrate chains O-glycosidically linked to serine or threonine residues are cleaved by acid catalyzed elimination reactions (11,13). Trifluoroacetolysis has also been used to release the carbohydrate portion of glycosylceramides (14), a reaction which is probably favoured by the allylic stabilization of an intermediate cation.

In this summary a comparison between acetolysis and trifluoroacetolysis will be made because of their inherent similarity. The two techniques will be treated in conjunction with general oligo- and polysaccharide analysis and synthetic applications.

2. ACETOLYSIS AND TRIFLUOROACETOLYSIS IN CARBOHYDRATE ANALYSIS

Acetolysis (15,16) is generally performed in mixtures of acetic anhydride, acetic acid and sulphuric acid. Other conditions have been used particularly in synthesis or in studies of mechanism. The sulphuric acid has sometimes been replaced by perchloric acid or different Lewis acids (ZnCl_2 , SnCl_4 , TiCl_4 , BF_3 , etc.). The acetic acid has in certain instances been excluded and in others non-polar solvents like carbon tetrachloride have been added. The reagent trifluoroacetic anhydride/acetic acid has also been used.

Acetolysis is a method of degradation which often gives information on sequence which may complement that from graded acid hydrolysis. In the fragmentation of oligo- and poly-saccharides by acetolysis (1-6)-linkages are particularly susceptible to cleavage, whereas they are relatively stable to acid hydrolysis (16). The terminal glycosidic bonds at the non-reducing end have been reported to be more stable to acetolysis than acid hydrolysis (17).

Anomerisation has been observed in acetolysis (18) as has epimerisation at C-2 of furanose residues having the hydroxyls at C-2 and C-3 in a cis arrangement (16).

Sialic acid residues (19), 2-acetamido-2-deoxy sugars situated at the reducing end of an oligo- or poly-saccharide (8,10,11) and reducing 2-deoxy sugars (8)' are especially prone to degradation. Glycosidically linked uronic acids are unstable as mixed anhydride derivatives but stable after lactone formation (20). Highly branched sugar residues in complex carbohydrate structures with few O-trifluoroacetyl groups will be

more readily solvolysed and degraded.

In contrast to results from trifluoroacetolysis the sialic acid linkages displayed an exceptionally high stability during the acetolysis of gangliosides (21).

Carbohydrates from natural sources sometimes contain non-sugar substituents. The behaviour of these under conditions of trifluoroacetolysis is also pertinent to synthetic applications and will be discussed in that context.

It would seem that as tools of carbohydrate analysis acetolysis and trifluoroacetolysis are useful in rather different ways. However, it might be rewarding to achieve extensive degradation in trifluoroacetolysis by using more severe conditions, i.e. higher temperature or higher acid concentration. Another possibility is to permethylate before trifluoroacetolysis. When a glycosidic bond is broken this engenders an O-trifluoroacetyl group which stabilizes the adjacent glycosidic bond. Cleavage of the glycosidic bond next to a reducing sugar residue, created in the process, would of course only lead to solvolysis. This method can be extended in that certain hydroxyl groups are protected before methylation. The protective groups would then be removed before trifluoroacetolysis. In many cases this would be unnecessary because of their inherent instability under the conditions employed. Trifluoroacetolysis may be performed either at a high temperature with a low TFA/TFAA ratio or at a lower temperature with a relatively high TFA/TFAA ratio.

3. FACTORS AFFECTING ACETOLYSIS AND TRIFLUOROACETOLYSIS.

MECHANISMS OF ANOMERISATION AND SOLVOLYSIS IN ACETOLYSIS

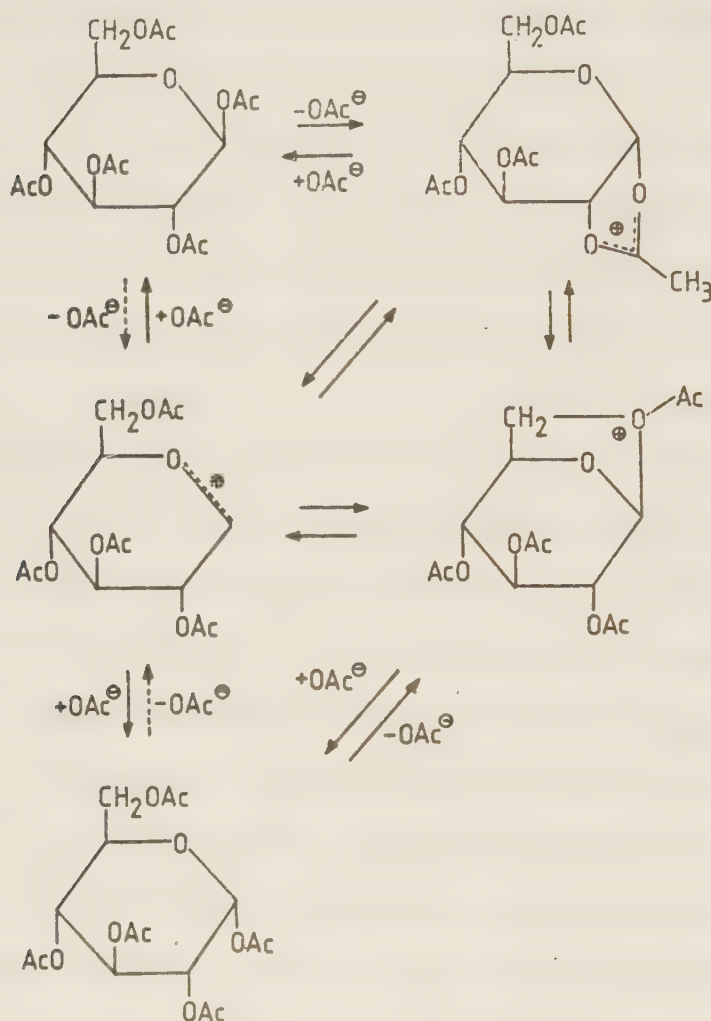
The attacking species in the reactions leading to anomerisation and solvolysis during acetolysis is an electrophile, presumably an acetylium ion or a Lewis acid catalyst depending on the composition of the reagent. Steric factors may be important in the release of strain in transition states or products as well as in obstructing the attack of the electrophile. Electronegative substituents may retard the rate of anomerisation and solvolysis. The rate of reaction and the product composition may also depend on anchimeric assistance by suitably positioned O-acetyl groups.

The same factors are presumably operable in trifluoroacetolysis but their relative importance may be reversed. Electronic effects are likely to be more significant in trifluoroacetolysis than in acetolysis, whereas anchimeric assistance may be negligible due to the electronegative character of the trifluoromethyl group. Thus different mechanisms may operate in trifluoroacetolysis and acetolysis of the same compound.

Most of the work on acetolysis has been aimed at elucidating the mechanisms of anomerisation of peracetylated aldopyranoses and anomerisation-solvolysis of glycopyranosides. Conditions of acetolysis have generally been selected so as not to cause any degradation of the solvolysis products.

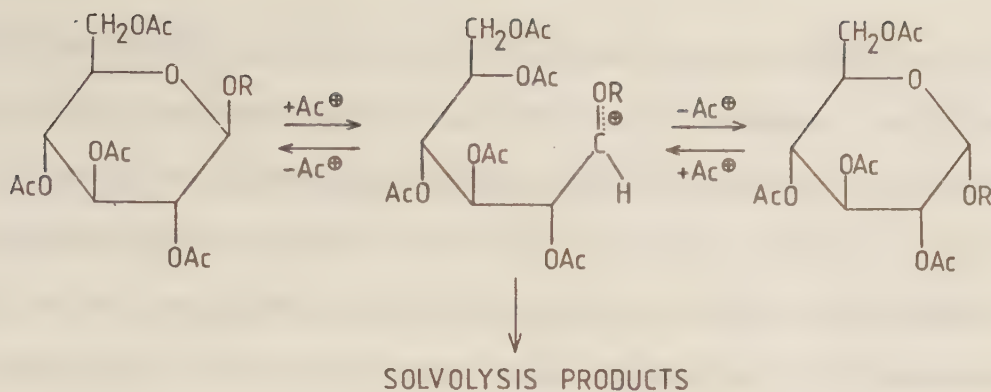
One of the earliest mechanisms for the anomerisation of D-glucopyranose pentaacetates in acetic anhydride, acetic acid and sulphuric acid was presented by Bonner (22). In this the acetic anhydride is protonated and participates in forming a

six-membered complex at the same time donating and accepting an acetoxy group. Lemieux (23) has suggested a mechanism for the anomerisation of peracetylated aldopyranoses' (Scheme 1). It is supported by isotope labelling experiments in which the exchange of the C-1 acetoxy group was established (24).



Scheme 1

Lindberg (25,26) has proposed ring-opening as a probable mechanism in the anomerisation-solvolysis of glycopyranosides (Scheme 2). Lemieux (27) presented a mechanism where the glycosidic oxygen is attacked and cleaved from C-1 forming a complex



Scheme 2

involving the ring oxygen. Participation, where possible, by the O-acetyl group at C-2 may facilitate these processes. The most important evidence in favour of the ring-opening mechanism is the isolation of peracetylated aldehydo compounds of which the acetolysis of methyl β -D-arabinopyranoside is an example (28).

Electronegative groups in the aglycone reduce the rate of solvolysis and anomerisation (25,26). Ballou and Rosenfeld (29) made a detailed study of the initial solvolysis rates for several disaccharides using acetic anhydride, acetic acid and sulphuric acid. In the D-glucobiose series the order of solvolysis for the β anomers decreased in the order (1-6)>>(1-3)>>(1-2)>(1-4) and for the α anomers in the order (1-6)>>(1-4)>>(1-3)>(1-2). Anchimeric assistance would explain the greater solvolysis rate found for the β anomers in comparison with the α anomers. The participation of the trans C-2 acetoxyl group in the β anomers would also explain why a strict order of aglycone stabilization is not observed. Rather extensive anomerisation was observed in some cases. The Lindberg mechanism of anomerisation and solvolysis for glycopyranosides was supported.

An interesting reaction indicating the initial steps in the mechanism in the degradation of reducing 2-acetamido-2-deoxy

sugars in trifluoroacetolysis was discovered when 2-acetamido-2-deoxy-D-glucose was dissolved in trifluoroacetic acid at room temperature (30). The ^1H -NMR spectrum showed the appearance of the 1,1-di-O-trifluoroacetyl aldehydo compound presumably formed via anchimeric assistance from the N-acetyl group in an acid catalyzed ring-opening. No such reactions were found with D-glucose or 2-amino-2-deoxy-D-glucose.

4. TRIFLUOROACETOLYSIS OF MODEL COMPOUNDS

4.1 General aspects

Several models have already been studied in trifluoroacetolysis (8,9,11-14). The aim of the now extended model compound studies is to assess and compare the stabilizing effects of differently positioned O-trifluoroacetyl groups and combinations of O-trifluoroacetyl groups in common carbohydrate structures. At the same time an indication is obtained of the effects that will govern the behaviour of oligo- and poly-saccharides.

Stability is expected to depend on conformation, anomeric configuration and configuration at other carbons. Furthermore, an O-methyl substituent in the glycosyl residue will not have exactly the same influence as a sugar substituent. An O-methyl group will be less rigid than a sugar chain aglycone and thus anomerisation might be more pronounced in the former case. The additional stabilization from aglycone O-trifluoroacetyl groups might be of importance.

Although the glycosidic bond between two sugar residues might be stable per se, elimination may occur because of the

instability of the aglycone, for example the degradation of 2-acetamido-2-deoxy sugars situated at the reducing end (11) under conditions where methyl 2-acetamido-2-deoxy- α -D-glucopyranoside is stable (12).

The trifluoroacetolysis experiments on the model compounds were conducted at 100°C for 48 h with ratios of trifluoroacetic acid to trifluoroacetic anhydride varying from 1/1 to 1/50 (v/v). These conditions were used because they effect complete transamidation. A sine qua non was of course that the investigated compounds displayed stabilizing effects. This was not the case for β -D-glucosides exhibiting the stabilizing effect of O-trifluoroacetyl groups exclusively in the aglycone. These compounds were all completely destroyed at 100°C even at TFA/TFAA 1/50 and 60°C was found to be suitable for the comparison of their relative stabilities.

A conceivable mechanism for the formation of the trifluoroacetylium ion is given in Fig. 1. Mechanisms involving attack from other species cannot be excluded.

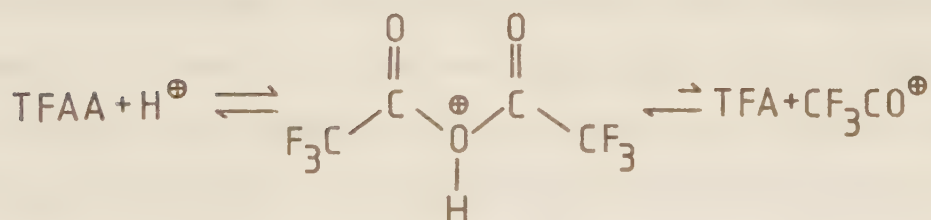


Fig. 1

4.2 Partially methylated methyl α -D-glucopyranosides

Partially methylated methyl α -D-glucopyranosides were subjected to trifluoroacetolysis at 100°C for 48 h together with a stable internal standard. Analysis was performed by GLC/GLC-MS after de-O-trifluoroacetylation, reduction and acetylation, i.e. no attempts were made to determine the structure of the solvolysis product(s).

Results. Methyl 2,3,4,6-tetra-O-methyl- α -D-glucoside was completely degraded even in TFA/TFAA 1/50 (v/v) leaving no trace of starting material, β anomer or solvolysis products. In contrast methyl 2,4,6-tri-O-methyl- α -D-glucoside, the most degraded of the glucosides containing free hydroxyl groups, was recovered in 59% yield under the same conditions, the total recovery including the β anomer (2%) and solvolysis products (26%) being close to 90%. The stability derived from a single O-trifluoroacetyl group in a particular position decreased in the order 2>>4>6>3. The greater stabilizing effect of the 2-O-trifluoroacetyl group is understandable as it is situated in a position β to both the ring oxygen and the glycosidic oxygen. In TFA/TFAA 1/1 (v/v) only the monosubstituted glucosides containing a free 2-position were recovered intact. Methyl 2-O-methyl- α -D-glucopyranoside and the glucosides with two free hydroxyls, one of which was in the 2-position, could be recovered in about 90% yield. The remaining dihydroxyl compounds and methyl 3,4,6-tri-O-methyl- α -D-glucoside were recovered in about 50% yield. A single O-trifluoroacetyl group in any other position than the 2-position was not enough to prevent complete solvolysis. In TFA/TFAA 1/50 the only compounds not to be recovered intact, apart from the permethylated glucoside already mentioned, were

methyl 2,3-di-O-methyl- α -D-glucopyranoside (92%) and the monohydroxyl glucosides except for the one with a free 2-position (59-79%). Anomerisation was found to occur in all cases where there was not total stability or complete solvolysis. The anomerisation did not in any case constitute more than 13%. Solvolysis products were stable from glucosides containing two or three hydroxyls under all conditions examined. In addition solvolysis products from the 2- and 4-monohydroxyl glucosides were stable in TFA/TFAA 1/15 and 1/50 and from the 6-monohydroxyl glucoside in TFA/TFAA 1/50.

Discussion of mechanism. The 2-O-trifluoroacetyl group should retard oxonium ion formation both of the ring and the glycosidic oxygens as well as hinder subsequent cleavage. The 4- and 6-positions can presumably only effectively protect the ring oxygen from electrophilic attack. Furthermore, if the ring oxygen in spite of the protection was transformed into an oxonium ion, both 4- and 6-O-trifluoroacetyl groups should enhance rate of cleavage between the ring oxygen and C-1. A 3-O-trifluoroacetyl group situated in a position γ to both the ring oxygen and the glycosidic oxygen exerts the weakest stabilizing influence. The effect is nevertheless rather dramatic in TFA/TFAA 1/50. The data obtained from trifluoroacetolysis of partially methylated methyl α -D-glucopyranosides appear to be most consistent with a mechanism in which electrophilic attack occurs principally at the ring oxygen although at this stage other mechanisms cannot be excluded.

4.3 Partially methylated methyl α -D-xylopyranosides and partially methylated D-xyloses

The most stable conformation of methyl α -D-glucopyranoside 4C_1 is also the most stable one for methyl α -D-xylopyranoside. The C-2, C-3 and C-4 hydroxyls of both compounds are all equatorially oriented in that conformation. These similarities prompted the investigation of the stability of partially methylated methyl α -D-xylopyranosides to trifluoroacetolysis. Partially methylated D-xyloses were also examined because of the occurrence of substituted D-xyloses in carbohydrate-protein linkages.

Results. The partially methylated methyl α -D-xylopyranosides were considerably less stable in TFA/TFAA 1/1 (v/v) than the corresponding partially methylated methyl α -D-glucopyranosides. In TFA/TFAA 1/50 (v/v) there was still a large difference between the recovery of the monohydroxyl xylosides and the corresponding glucosides whereas the difference was less pronounced between the respective dihydroxyl compounds. Methyl 2,3,4-tri-O-methyl- α -D-xyloside was completely degraded in TFA/TFAA 1/50 as was the corresponding glucoside. However, the recoveries of the monohydroxyl compounds did not indicate any large stabilizing effect of a single O-trifluoroacetyl group. The 4-position was most important and the stabilizing effect decreased in the order 4>2>3 in TFA/TFAA 1/50. In TFA/TFAA 1/1 all the disubstituted xylosides were completely destroyed as well as any solvolysis products. When two hydroxyl groups were free the 2-position had a dominating influence. The two compounds containing a hydroxyl in the 2-position were recovered in about 45% yield in TFA/TFAA 1/1. In TFA/TFAA 1/50 they could

be recovered virtually intact. Only a few per cent of methyl 2-O-methyl- α -D-xylopyranoside was recovered in TFA/TFAA 1/1, whereas about 90% was recovered in TFA/TFAA 1/50. Methyl α -D-xylopyranoside was stable in TFA/TFAA 1/50 and recovered in about 90% in TFA/TFAA 1/1. Anomerisation did not occur to any great extent although methyl 3-O-methyl- α -D-xylopyranoside yielded 9% of the β anomer in TFA/TFAA 1/1. The yields of solvolysis products from xylopyranosides undergoing degradation during trifluoroacetolysis were low. For the partially methylated D-xyloses a free 4-position was found to be by far the most important in preventing degradation. This was true for both mono- and di-substituted D-xyloses.

Discussion of mechanism. The larger stabilizing effect of a single O-trifluoroacetyl group in the 4-position can be explained in terms of the greater accessibility of the ring oxygen in the xylosides compared with the glucosides, owing to the steric hindrance of the substituted 5-C carbinol group in the latter. However the difference in the stabilizing effect between the 2- and 4-positions was rather small. In the monosubstituted xylosides the 2-position becomes the dominating influence and may indicate that for those substances it is more important to prevent cleavage by ring-opening than suppress oxonium ion formation. The partially methylated D-xyloses would be expected to form predominantly pyranose or furanose pertrifluoroacetates - at least in the initial stage of trifluoroacetolysis. The low recovery of solvolysis products from some of the xylosides can be rationalized by the assumption that open-chain solvolysis products were formed. Xyloses with free 4-positions acquire greater resistance to degradation, and thus there is a possi-

bility that for pentoses furanose pertrifluoroacetates are the most stable in trifluoroacetolysis. Pyranose pertrifluoroacetates of pentoses might be susceptible to electrophilic attack on the ring oxygen and subsequent ring-opening would possibly lead to open-chain aldehydo pertrifluoroacetates.

4.4 Partially methylated methyl α -L-arabinofuranosides

Partially methylated methyl α -L-arabinofuranosides were subjected to trifluoroacetolysis under the same conditions as described earlier for the pyranosides. Steric strain in furanosides is predominantly caused by eclipsing of the 1,2-cis substituents. This can be lessened by shifting one or two appropriate carbon atoms out of plane to adopt envelope or twisted conformations. Non-bonded interactions similar to those of 1,3-diaxial interactions in pyranosides may also be present. The most stable conformations of methyl α -L-arabinofuranoside are two twist forms in which there is little steric strain (31).

Results. In contrast to the previously examined permethylated glycosides trifluoroacetolysis of methyl 2,3,5-tri-O-methyl- α -L-arabinoside in TFA/TFAA 1/50 (v/v) afforded a trace of undegraded solvolysis products. In TFA/TFAA 1/1 (v/v) methyl 3,5-di-O-methyl- α -L-arabinoside was recovered in a yield of 5%, whereas the other monohydroxyl arabinosides were completely degraded leaving only traces of solvolysis products. In TFA/TFAA 1/50 the stability of a ring containing a single O-trifluoroacetyl group decreased in the order 2>3>5. The amounts recovered were 70%, 41% and 8%, respectively. A single O-trifluoroacetyl group had a larger stabilizing effect in the arabinofuranosides than in the xylopyranosides under these conditions. Only the 2-posi-

tion in the arabinosides and xylosides is directly comparable, the 5-position being primary and the 3-position being situated β to the ring oxygen in the arabinosides. In TFA/TFAA 1/1 starting material was recovered from the 3-O-methyl (20%) and the 5-O-methyl (27%) arabinosides, whereas in TFA/TFAA 1/50 they were essentially stable. There was no recovery of the 2-O-methyl arabinoside in TFA/TFAA 1/1, but in TFA/TFAA 1/50 the recovery was 92%. In comparison with the monosubstituted xylopyranosides the arabinofuranoside analogues were less stable in TFA/TFAA 1/1, whereas they exhibited similar stabilities in TFA/TFAA 1/50. The recovery of methyl α -L-arabinofuranoside was 68% in TFA/TFAA 1/1, the remainder being anomerized (20%) or solvolyzed (14%), but in TFA/TFAA 1/50 this compound was stable as expected. Anomerisation was also observed for the mono- and disubstituted arabinosides, but in no case did the amount of recovered β anomer exceed 9%. Solvolysis products were recovered quantitatively in TFA/TFAA 1/50 with two exceptions. One has already been mentioned and the other was methyl 2,3-di-O-methyl- α -L-arabinofuranoside which yielded solvolysis products that were almost stable. Solvolysis products from the monosubstituted arabinosides were almost stable in TFA/TFAA 1/1, whereas the recovery of solvolysis products from the disubstituted arabinosides was only 2-12%.

Discussion of mechanism. A single O-trifluoroacetyl group had a larger influence on stability in the 2-position than in any other position for the arabinofuranosides and the glucopyranosides but not for the xylopyranosides. This is presumably due to the bulky groups adjacent to the ring oxygens in the arabinosides and the glucosides hindering electrophilic attack.

O-Trifluoroacetyl groups in the 3- or 5-positions of the arabinosides should protect the ring oxygen from ionisation, but enhance rate of ring-opening. Their ability to protect the glycosidic oxygen should be small.

4.5 The stabilizing influence of O-trifluoroacetyl groups in the aglycone of β -D-glucopyranosides in preventing solvolysis

It was clear from the trifluoroacetolysis of oligosaccharides (9) that O-trifluoroacetyl groups in the aglycone did not have any large destabilizing effect. A stabilizing influence was later indicated by the difference in stability of 3- and 6-linked sialic acid residues (19), the 3-linked compound being more stable. The data were however not sufficient to give an idea of the size of the effect. To evaluate the influence on stability exerted by differently positioned O-trifluoroacetyl groups in the aglycone β -D-glucosides fully methylated in the ring positions and with aglycone structures containing one or two free hydroxyls were synthesized and subjected to trifluoroacetolysis. At 100°C all these compounds were completely destroyed in TFA/TFAA 1/1 and 1/50 and their relative stabilities were therefore examined at 60°C. A hydroxyl in the 6-position of the glucosyl residue permitted the recovery after trifluoroacetolysis at 100°C, of glucosides containing aglycone hydroxyl groups. In this way the additional stability derived from O-trifluoroacetyl groups in the aglycone could be examined under conditions normally employed in trifluoroacetolysis. Compounds having these features were synthesized and subjected to trifluoroacetolysis at 100°C for 48 h.

Results. As can be seen from Table 1 there was little

TABLE 1 Trifluoroacetolysis of methyl 2,3,4,6-tetra-O-methyl- β -D-glucoside (Me 2,3,4,6-OMe-Glc) and compounds 1-4 in TFA/TFAA (1/1 and 1/50) at 60°C for 48 h.

Compound	Recovery (mol%) ^a	
	1/1	1/50
Me 2,3,4,6-OMe-Glc	6	46
<u>1</u>	4	77
<u>2</u>	22	85
<u>3</u>	30	93
<u>4</u>	5	74

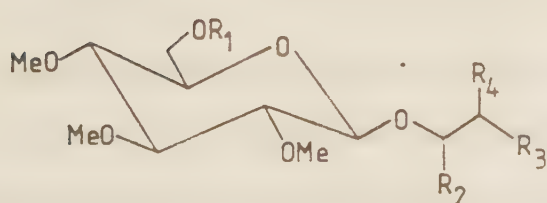
^a Determined after de-O-trifluoroacetylation, reduction (NaBD₄) and acetylation.

TABLE 2 Trifluoroacetolysis of methyl 2,3,4-tri-O-methyl- β -D-glucoside (Me 2,3,4-OMe-Glc) and compounds 5 and 6 in TFA/TFAA (1/1, 1/4 and 1/50) at 100°C for 48 h.

Compound	Recovery (mol%) ^a		
	1/1	1/4	1/50
Me 2,3,4-OMe-Glc	-	4 (20) ^b	47 (36)
<u>5</u>	-	6	59
<u>6</u>	1	24	80

^a Determined after de-O-trifluoroacetylation, reduction (NaBD)₄, and acetylation.

^b Values in parenthesis represent anomerized product.



	R ₁	R ₂	R ₃	R ₄
<u>1</u>	Me	H	H	OH
<u>2</u>	Me	H	OH	CH ₂ OH
<u>3</u>	Me	CH ₂ OH	H	OH
<u>4</u>	Me	H	OMe	CH ₂ OH
<u>5</u>	H	H	H	OH
<u>6</u>	H	CH ₂ OH	H	OH

difference in the stability in TFA/TFAA 1/50 (v/v) at 60°C derived from aglycones containing a single O-trifluoroacetyl group adjacent to the glycosidic bond and an O-trifluoroacetyl group one carbon atom removed from the glycosidic bond. Under these conditions there is a small increase in stability between the different compounds that follows the expected order. In TFA/TFAA 1/1 at the same temperature only compounds 2 and 3 show an increased stability. The stabilizing effect from aglycone O-trifluoroacetyl groups, although per se rather small, might in conjunction with other effects be very important in providing additional stabilization (see Table 2).

Anomerisation was only seen in the methyl glucosides. In the case of methyl 2,3,4-tri-O-methyl- β -D-glucoside, a large amount of α anomer was recovered in TFA/TFAA 1/4 and 1/50. In the analysis peracetylated glycerol residues and solvolysis products were also detected.

Discussion of mechanism. Aglycone O-trifluoroacetyl groups are not likely to be effective in preventing oxonium ion formation at the ring oxygen. On the other hand they would reduce the rate of reaction of the subsequent ring-opening. Although electrophilic attack on the glycosidic oxygen would be hindered, the rate of cleavage of the oxonium ion resulting from such an attack would be enhanced. However, when the ring oxygen was protected by a 6-O-trifluoroacetyl group the increase in stability that could be attributed to O-trifluoroacetyl groups in the aglycone was more striking than would have been expected from the results of Table 1. This may indicate that the ring oxygen is preferentially attacked in the solvolysis of these glucosides.

5. SYNTHETIC APPLICATIONS

The reagents trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) have many useful applications in organic chemistry and biochemistry. TFA is a strong acid and may replace aqueous mineral acids in many reactions. It has excellent solvent properties and is volatile, which permits an easy work-up by evaporation without fear of increased acid concentration. TFAA is a powerful acylating agent because it generates reactive mixed anhydrides with other acids. An example of the usefulness of this reaction is the esterification of the highly sterically hindered mesitoic acid (32). O-Trifluoroacetyls have been used as protective groups since they may be removed selectively from certain positions (33-35). Primary O-trifluoroacetyl groups are for example more easily cleaved than secondary ones. Migrations occur in basic media, even during acetylation with acetic anhydride and pyridine, but not in acidic (36).

A synthetic use of acetolysis is to prepare different oligosaccharides via their peracetylated derivatives from polysaccharides (37). Preparative changes of anomeric configuration (38), by means of Lewis acid catalysts, has been achieved on a preparative scale with glycosides, peracetylated glycosyl halides and peracetylated pyranoses or furanoses.

Complete or selective cleavage of acetals and ketals can be accomplished by acetolysis (15). Methylene acetals occupying a primary and a secondary position are cleaved to yield a primary acetate and a secondary acetoxymethyl ether in acetic anhydride, acetic acid and sulphuric acid. When acetolysis is conducted using acetic acid and trifluoroacetic anhydride, the anion is

the trifluoroacetate ion and the trifluoroacetoxymethyl ether formed is easily cleaved to yield a free hydroxyl group leaving the O-acetyl group intact. Isopropylidene derivatives yield diacetates from both systems. Benzylidene acetals give diacetates on acetolysis. Trifluoroacetolysis has also been used to remove benzylidene protecting groups under different conditions, some of them rather mild (39).

Aqueous trifluoroacetic acid (90%) at room temperature hydrolyses isopropylidene and benzylidene groups (40). The rapid reaction leaves many common functional groups unchanged.

Benzyl blocking groups may be cleaved by acetolysis (15). Primary benzyl groups react more readily than secondary ones. Trifluoroacetic acid at room temperature was found to cleave aromatic benzyl ethers (41). Benzyl ethyl ether and benzyl n-butyl ether were reported to be unaffected under the same conditions. However, the benzyl group of the secondary alcohol in methyl 2-O-benzyl-3,4,6-tri-O-methyl- α -D-glucoside was quantitatively removed by trifluoroacetic acid at room temperature after 15 h (19). The glycosidic linkage was not attacked. Trifluoroacetolysis conditions have also been used to remove benzyl blocking groups, the idea being that the engendered O-trifluoroacetyl groups will protect the glycosidic bonds (19). A high acid concentration appears to be more important than a high temperature, particularly in compounds with multiple benzyl groups or several free positions. In these compounds, benzyl groups are expected to be stabilized by adjacent O-trifluoroacetyl groups. Reaction times have varied between 0.5 and 6 h and the yields from differently substituted glycosides have ranged from 70 to 100.

Trityl groups have been cleaved in TFAA/CHCl₃ 1/1 (v/v) at room temperature for 10-30 min being replaced by O-trifluoroacetyl groups (19). This reaction may be due to the traces of acid generated by the alcohol (1% in chloroform).

O-Acetyl groups have been shown to be stable in trifluoroacetolysis using TFA/TFAA 1/50 (v/v) at 100°C for 48 h (20). The recovery from TFA/TFAA 1/1 was less than quantitative, but at lower temperatures acetates may be completely stable at even higher acid concentrations.

The mild conditions of the de-O-tritylation and the stability of O-acetyl groups has permitted the synthesis of a trityldextran in which all primary positions are tritylated with a minimum of secondary trityl groups (39). To achieve the complete tritylation of all primary positions an excess of reagent has to be used (42). Under such conditions many secondary positions are also tritylated. Acetylation, de-O-tritylation, de-O-trifluoroacetylation and a new tritylation improves the ratio of primary to secondary positioned trityl groups. This sequence of reactions is repeated until virtually all secondary positions are acetylated or free. Finally the O-acetyl groups are removed. In the resulting trityldextran all non-reducing terminal D-glucose residues should have been converted into 6-O-trityl-D-glucose residues. Such a modified dextran is of interest for studying the immunochemistry of the dextran-antidextran system.

The rather mild conditions used for trifluoroacetolysis and, above all, the stabilizing influence of O-trifluoroacetyl groups on the glycosidic bonds have great potential in synthetic operations on carbohydrate macromolecules.

6. STRUCTURAL INVESTIGATION OF THE CARBOHYDRATE PORTION OF HUMAN ANTITHROMBIN III

Human antithrombin III (43-47) is a glycoprotein involved in the regulation of blood coagulation. It is considered to be a major factor in reducing thrombin activity. This inhibition is potentiated by the polysaccharide heparin. As antithrombin III binds to heparin it can be purified from plasma by affinity chromatography on heparin-agarose gels. The molecular weight of antithrombin III is about 60,000 daltons. It is slightly acidic and has a carbohydrate content of 10-15% by weight.

The homogeneity of the preparation used was established by gel filtration and electrophoresis.

The relative molar proportions of the sugar constituents in human antithrombin III as revealed by sugar analysis (48,49) were: D-Man (3.1), D-Gal (2.0), D-GlcNAc (3.7), and NeuNAc (1.9). Thereafter methylation analysis (50,51) was carried out before and after desialylation of the glycoprotein. The results are presented in Table 3.

The removal of sialic acid residues was carried out with neuraminidase (Vibrio cholerae) and by mild acid hydrolysis (52). On the grounds of the specificity of the enzyme and the two methylation analyses, the sialic acids should be terminal and α -linked to the galactose residues in the 6-position.

Antithrombin III had thus been shown to contain:

α -NeuNAc-(2-6)- <u>D</u> -Galp-(1-	2 residues
-4)- <u>D</u> -GlcNAcp-(1-	4 residues
-2)- <u>D</u> -Manp-(1-	2 residues
-3)- <u>D</u> -Manp-(1-	1 residue
6	

TABLE 3 Methyl ethers obtained from hydrolysates of methylated antithrombin III and desialylated antithrombin III

Sugars	T-value ^a SE-30	Relative molar proportions ^b Antithrombin / Antithrombin
2,3,4,6-O-Me-D̄-Gal	1.07	<0.05 (0) ^c 2.1 (2)
2,3,4-O-Me-D̄-Gal	1.82	1.9 (2) <0.05 (0)
3,4,6-O-Me-D̄-Man	1.45	2.1 (2) 2.0 (2)
3,6-O-Me-D̄-Man	2.40	1.0 (1) 1.0 (1)
3,6-O-Me-D̄-GlcN(Me)Ac	4.15)	+ (4) ^d + (4) ^d
3,6-O-Me-D̄-GlcNac	4.32	

^a Retention times of the corresponding alditol acetates relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D̄-glucitol.

^b Desialylated.

^c Figures in parentheses represent nearest integer.

^d The relative molar proportions were calculated from the sugar analysis (see text).

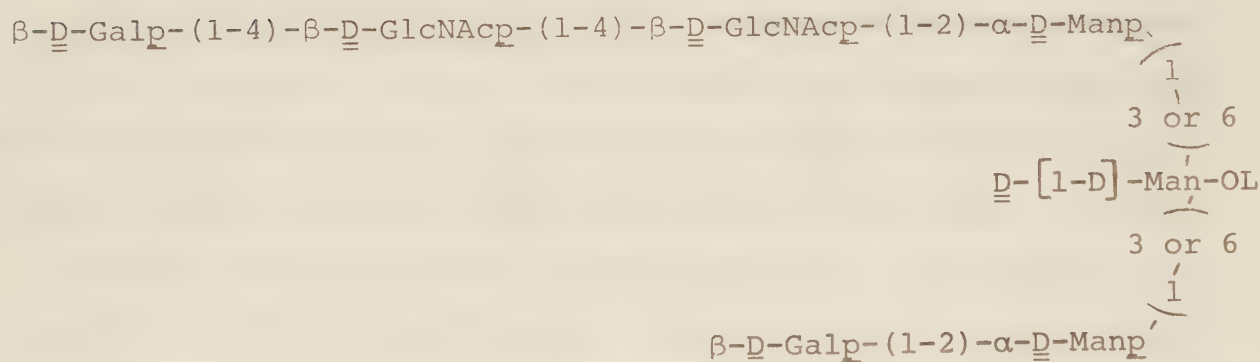
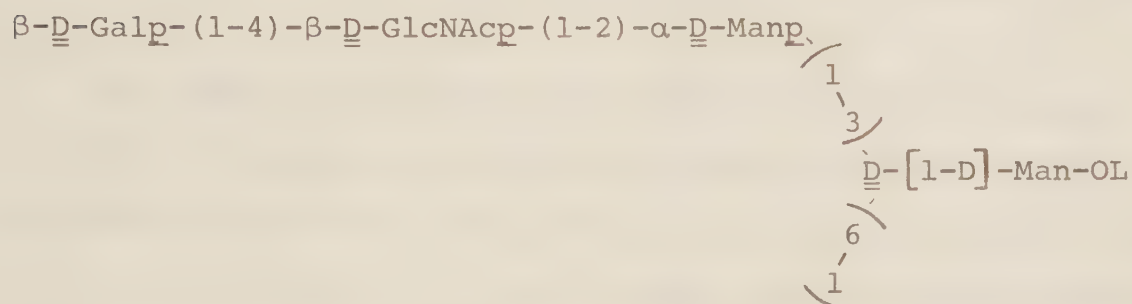
Trifluoroacetolysis [TFA/TFAA, 1/1 (v/v), 100°C, 48 h] would degrade the glycoprotein in the following manner: The protein part would be degraded by transamidation and release the carbohydrate chains (10,11). A sequence of GlcNAc residues situated at the reducing end would be completely degraded (8,10,11). Glycosidically linked GlcNAc residues which are not part of such a sequence would be transamidated (12). The terminal 6-linked NeuNAc residues would be quantitatively cleaved (19).

The operations used in the work-up lead to removal of O-trifluoroacetyl groups, replacement of N-trifluoroacetyl groups by N-acetyl groups and reduction (NaBD_4). The resulting oligosaccharide was purified by gel filtration. Sugar analysis showed that no sialic acid residues were left as expected. It also revealed that two GlcNAc residues had been degraded. Methylation analysis showed that the branched mannose residue was situated at the reducing end of the oligosaccharide obtained after the trifluoroacetolysis degradation.

In order to determine the anomeric configuration of the linkages in the oligosaccharide thus obtained it was subjected to chromium trioxide oxidation (53). Free hydroxyl groups are attacked by the reagent but ester and amide bonds are stable and therefore the material is acetylated before oxidation. All the sugars present should have ${}^4\text{C}_1$ conformations and hence the β -linkages should be attacked to yield 5-hexulosonates, whereas α -linkages should be essentially stable. Sugar analysis gave only traces of D-Gal and D-GlcNAc. D-Man and D-[1-D]-Man-OL were present in the ratio 2:1 in the oligosaccharide. Treatment with base split the ester linkages in the 5-hexulosonates and after permethylation the presence of the trisaccharide α -D-Manp-

(1-3)-[α -D-Manp-(1-6)-]-D-[1-D]-Man-OL was established by comparison with an authentic sample in GLC/GLC-MS.

The oligosaccharide isolated after the trifluoroacetytic degradation may thus be assigned the structure $\sim$ 1, $\sim$ 2 or $\sim$ 3.



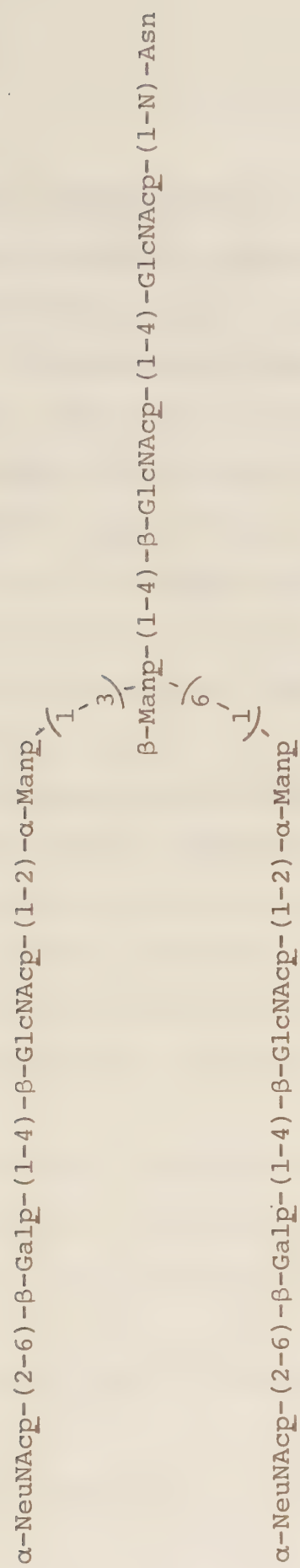
Provided that the carbohydrate chains are homogeneous a Smith degradation (54) of antithrombin III should now make it possible to distinguish between the structures $\sim$ 1-3. After the reduction (NaBH_4) and the mild acid hydrolysis used in the Smith

degradation procedure a dialyzable fraction was isolated. This was reduced (NaBD_4) and permethylated. On analysis by GLC-MS the only sugar derivative to be detected was β -D-GlcNAcp-(1-2)-[1-D]-glycerol which was identified by comparison with an authentic sample. This establishes structure 1 as the correct one.

The nondialyzable fraction was subjected to sugar and methylation analysis. D-Man and D-GlcNAc were present in the relative molar proportions 1:2, the D-Man residue being terminal and the D-GlcNAc residues substituted in the positions 1 and 4. Finally the anomeric configuration of the linkages between these three innermost sugars bound to the protein was investigated.

When trifluoroacetolysis of desialylated antithrombin III was carried out using TFA/TFAA 1/100 (v/v) (100°C , 48 h), sugar analysis demonstrated that little or no degradation of the D-GlcNAc residues was taking place. In the work-up the released carbohydrate chains were reduced (NaBH_4) and acetylated. Chromium trioxide oxidation and subsequent sugar analysis (using NaBD_4 in the reduction) gave D-Man and D-GlcNAc-OL in the relative molar proportions 2:1 and only a trace of D-Gal. This finding demonstrates that the two linkages between the three innermost sugars should both have the β configuration.

The molecular weight of human antithrombin III in relation to the carbohydrate content and the analytical data presented here show that, in this glycoprotein, four identical carbohydrate chains should be present having the structure depicted on the opposite page (the linkage to asparagine is hypothetical).



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Note

The stability of partially methylated methyl α -D-glucopyranosides towards trifluoroacetolysis

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Under trifluoroacetolysis conditions that will effect transamidation¹, most glycosides² and reducing sugars³ are stable, being converted into their pertrifluoroacetylated derivatives. These findings have enabled us to develop procedures for the isolation of *O*- and *N*-glycosylically-linked carbohydrate chains in glycoproteins⁴ and glycopeptides⁵. It has also been demonstrated that the glycosidic bond to the sphingosine moiety in glycolipids can be cleaved by trifluoroacetolysis⁶. A prerequisite for the stability of glycosidic bonds in oligo- and poly-saccharides is that enough *O*-trifluoroacetyl groups are introduced close to the glycosidic oxygens, in order to withdraw electrons and thereby prevent solvolysis.

We now report studies of the stability towards trifluoroacetolysis of glucosides having a limited number of free hydroxyl groups.

Partially methylated methyl α -D-glucopyranosides having hydroxyl groups in all possible combinations were treated with trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) in proportions varying from 1:1 to 1:50 at 100°C for 48 h. The reaction mixtures were analysed after *O*-detrifluoroacetylation, reduction (NaBD₄), and acetylation. The results are summarized in Table I.

The reagent TFA/TFAA contains, in addition to TFA and TFAA, the ionic species CF_3CO^+ and CF_3COO^- formed by disproportionation of TFAA. When treated with the reagent, free hydroxyl groups are rapidly trifluoroacetylated. *O*-Trifluoroacetyl groups are strongly electron-attracting and, when positioned close to the acetal oxygen atoms of a glycoside, render the glycosidic bond stable towards solvolysis by TFA/TFAA. However, if the hydroxyl groups close to the glycosidic bond are blocked, the glycoside may be solvolysed by TFA/TFAA, and the pertrifluoroacetylated sugar formed may be further degraded by acid-catalysed elimination reactions. Anomerisation may also occur.

As can be seen from Table I, all of the partially methylated methyl α -D-glucopyranosides having HO-2 unsubstituted are stable in 1:50 TFA/TFAA. When HO-2 is blocked by a methyl group, minor degradation takes place with the 2,3-di-*O*-methylglucoside in 1:50 TFA/TFAA. Under the same conditions, the trimethylated

TABLE I

TRIFLUOROACETOLYSIS OF PARTIALLY METHYLATED METHYL α -D-GLUCOPYRANOSIDES USING TFA/TFAA (1:1, 1:4, 1:15, AND 1:50) AT 100°C FOR 48 h

Location of methyl group(s)	Recovery (mol%) ^a									
	Starting material					Anomerised product				
	1:1	1:4	1:15	1:50		1:1	1:4	1:15	1:50	
						Free sugar				
	1:1	1:4	1:15	1:50		1:1	1:4	1:15	1:50	Total recovery
2	89	n.d. ^b	n.d.	103	— ^c	2	n.d.	n.d.	—	103
3	97	n.d.	n.d.	99	—	<1	n.d.	n.d.	—	97
4	100	n.d.	n.d.	102	—	<1	n.d.	n.d.	—	100
6	102	n.d.	n.d.	103	—	<1	n.d.	n.d.	—	102
2,3	52	71	88	92	4	31	14	5	3	102
2,4	49	75	94	102	<1	34	13	5	<1	96
2,6	61	84	99	97	<1	28	8	3	<1	100
3,4	87	99	103	101	—	6	1	—	—	98
3,6	91	97	98	98	—	5	<1	—	—	99
4,6	92	100	103	97	—	5	<1	—	—	99
2,3,4	—	6	51	66	9	—	13	28	23	—
2,3,6	1	17	64	79	3	21	41	29	16	22
2,4,6	—	1	26	59	2	—	15	31	26	—
3,4,6	45	81	94	97	<1	33	10	3	<1	82
2,3,4,6	—	n.d.	n.d.	—	—	—	n.d.	n.d.	—	—

^aDetermined after *O*-de trifluoroacetylation, reduction (NaBD₄), and acetylation; ^bn.d., not determined; ^c—, not detectable.

glucosides having a hydroxyl group at C-3, C-4, or C-6 could only be recovered in 60–80% yield, the remainder being anomerised or solvolysed. The total recovery of the 2,4,6-tri-*O*-methylglucoside was not quantitative even in 1:50 TFA/TFAA, demonstrating that the solvolysed products were further degraded, probably *via* acid-catalysed eliminations. When methyl 2,3,4,6-tetra-*O*-methyl- α -D-glucoside was treated with 1:50 TFA/TFAA, no glucoside or free sugar could be recovered. This finding is expected as the fully methylated glucoside should be rapidly solvolysed and the resulting trifluoroacetate further degraded *via* acid-catalysed elimination reactions.

When the partially methylated glucosides were treated with 1:1 TFA/TFAA, all those containing three hydroxyl groups were stable, except for the one having the 2-position blocked, which underwent ~10% anomerisation. For glucosides containing two hydroxyl groups, there was also a marked difference in stability in 1:1 TFA/TFAA between those having the 2-position blocked and those having a free hydroxyl group at C-2. When only one hydroxyl group was unsubstituted, all glucosides were completely solvolysed, except for a trace of that having HO-4 unsubstituted and ~50% of that having HO-2 unsubstituted. The solvolysed products were also degraded further and, after *O*-detrifluoroacetylation, the free sugar could only be obtained from the trimethylated glucosides having HO-2 or HO-4 unsubstituted.

The foregoing results suggest that glycosidic bonds of oligosaccharides and polysaccharides containing hexopyranose residues should be stable in 1:50 TFA/TFAA, provided that no hexose residue is more than disubstituted. 3,4,6-Trisubstituted hexopyranose residues, with HO-2 unsubstituted, should also be stable. Since the glycosidic bonds in oligo- and poly-saccharides may be further stabilised by the hydroxyl groups of the aglycon residue, many of them should be stable even in 1:1 TFA/TFAA.

EXPERIMENTAL

Concentrations were performed at reduced pressure with bath temperatures not exceeding 40°C. G.l.c.-m.s. was performed with a combined gas chromatograph-mass spectrometer (Varian MAT 311A). Separations were performed at 180°C on glass-capillary columns (25 m $\times$ 0.25 mm) wall-coated with SE-30 or OV101 (LKB-Products, Sweden). Quantitative analyses with these columns were performed with a Perkin-Elmer 3920 gas chromatograph equipped with a flame-ionisation detector.

Partially methylated methyl α -D-glucopyranosides⁷ were obtained by standard synthetic procedures. The identity and purity of the compounds were established by g.l.c.-m.s.⁸ of the corresponding acetates.

Trifluoroacetolysis experiments. — The partially methylated methyl α -D-glucopyranoside (15 mg) and 10 mg of methyl α -D-glucopyranoside or methyl α -D-mannopyranoside (as stable internal standard⁹) were dissolved in methanol (5 ml), and 1 ml of the mixture was analysed by g.l.c.-m.s. after acetylation, to obtain response factors. Other portions (1 ml) were dried, and solutions of the residues in TFA/TFAA (1:1, 1:4, 1:15, and 1:50; 4 ml) were then heated in sealed glass tubes at 100°C for 48 h

(caution: corrosive mixture under pressure). The mixtures were then cooled to room temperature and evaporated to dryness. The residues were dissolved in methanol (2 ml), and the solutions were evaporated to dryness. The residues were then dissolved in ethanol-water (2:1; 2 ml) and reduced with sodium borodeuteride (10 mg). The reduced products were acetylated, and analysed by g.l.c.-m.s. The identity of each component was established by its mass spectrum⁸ and retention time in g.l.c., in comparison with authentic samples.

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II

The stability of partially methylated methyl α -D-xylopyranosides and partially methylated D-xyloses towards trifluoroacetolysis

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Trifluoroacetolysis is a reaction which is carried out in mixtures of trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) in various proportions and at different temperatures. 2-Acetamido-2-deoxy functions in sugar residues are converted into 2-deoxy-2-trifluoroacetamido groups, by trifluoroacetolysis, using TFA/TFAA in proportions varying from 1:1 to 1:50, at 100°C for 48 hours¹. Under these conditions most free sugars² and glycosides³ are stable due to the strong inductive effects exerted by the O-trifluoroacetyl groups which are rapidly formed by the action of TFA/TFAA on hydroxyl groups in the sugar moieties. Peptide bonds are cleaved by transamidation and thus proteins and the protein part of glycoproteins will be degraded whereas the carbohydrate portion of glycoproteins remains virtually intact apart from some degradation at the reducing end⁴. N-Glycosidically linked carbohydrate chains are cleaved from glycoproteins by transamidation⁴ and O-glycosidically linked carbohydrate chains (to serine or threonine) are split off by acid catalyzed elimination^{4,5}. Trifluoroacetolysis can also be used for specific cleavage of the glycosidic linkage between the oligosaccharide and ceramide portions in glycolipids⁶. In order to study the stabilizing effect of O-trifluoroacetyl groups in

preventing solvolysis of glycosides a detailed investigation of trifluoroacetolysis of partially methylated methyl α -D-glucopyranosides⁷ has been made. This study is now extended in this communication with trifluoroacetolysis studies on partially methylated methyl α -D-xylopyranosides and partially methylated D-xyloses.

The model compounds were treated with TFA/TFAA in the proportions 1:1 and 1:50 for 48 h together with a stable internal standard (xylitol or methyl α -D-glucopyranoside).

After de-O-trifluoroacetylation, reduction, and acetylation the resulting mixtures were analyzed by g.l.c.-m.s.⁸ (Tables I and II). As can be seen from Table I methyl 2,3,4-tri-O-methyl-

TABLE I

TRIFLUOROACETOLYSIS OF PARTIALLY METHYLATED METHYL α -D-XYLOPYRANOSIDES USING TFA/TFAA

Location of methyl group(s)	Recovery (mol%) ^a					
	Starting material		Free sugar		Total recovery	
	1:1	1:50	1:1	1:50	1:1	1:50
0	92	99	4	- ^b	96	99
2	4	91	18	9	22	100
3	43	97	18	<1	70 ^c	97
4	47	97	8	1	55	98
2,3	-	25	4	8	4	33
2,4	-	2	-	<1	-	2
3,4	-	12	-	2	-	14
2,3,4	-	-	-	-	-	-

^a Determined after de-O-trifluoroacetylation, reduction (NaBD₄) and acetylation.

^b = not detected.

^c This value includes 9% of anomerised product.

TABLE II

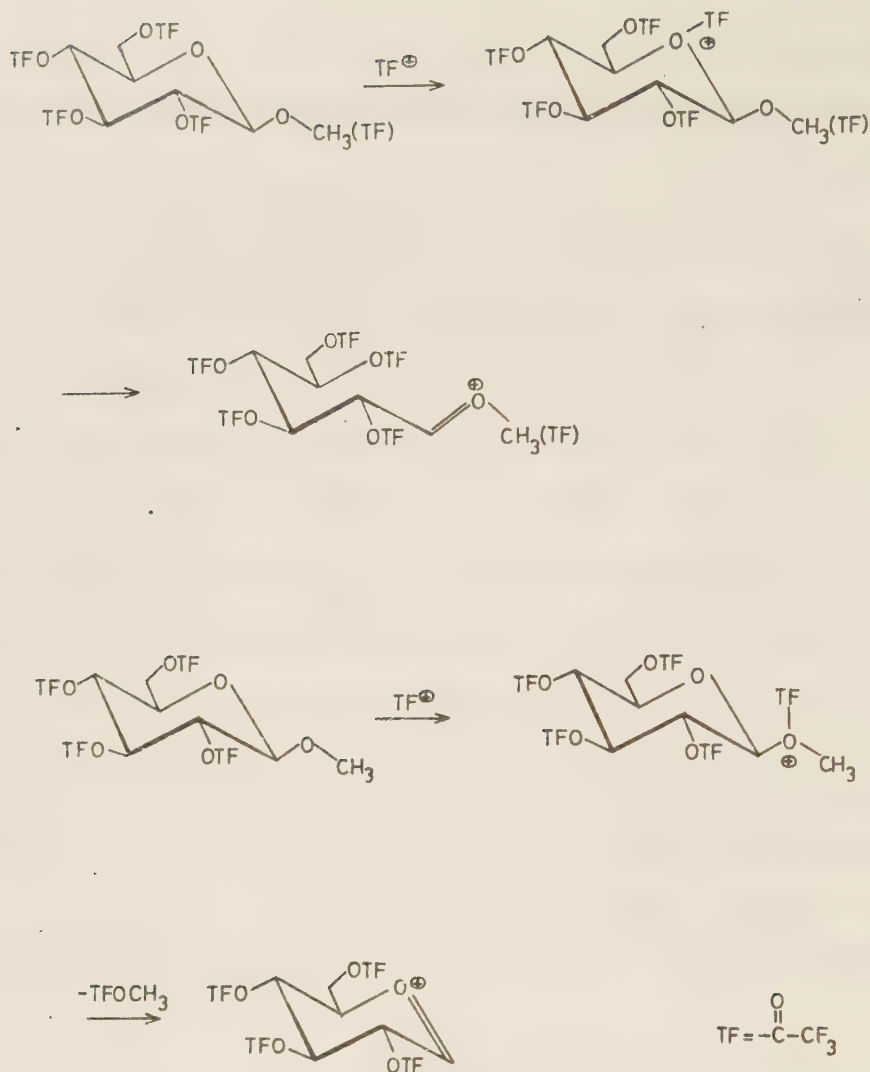
TRIFLUOROACETOLYSIS OF PARTIALLY METHYLATED D-XYLOSES
USING TFA/TFAA

Location of methyl group(s)	Recovery (mol%) ^a	
	Starting material 1:1	1:50
0	97	100
2	71	98
3	62	94
4	6	65
2,3	12	60
2,4	- ^b	<1
3,4	-	3
2,3,4	-	-

^a Determined after de-O-trifluoroacetylation, reduction (NaBD₄) and acetylation.

^b = not detected.

α -D-methyl- α -D-xyloside was completely destroyed at both trifluoroacetolysis conditions used presumably via solvolysis and acid catalyzed elimination reactions occurring on the pertrifluoroacetate (cf. Table II). With one free hydroxyl group some unchanged glycoside could be recovered after trifluoroacetolysis at the 1:50 conditions. The stabilizing effects were somewhat larger for a 4-O-trifluoroacetyl group than for a 2-O-trifluoroacetyl group. A trifluoroacetyl group in the 3-position had a small protective effect. The first step in the solvolysis reaction, by trifluoroacetolysis, on the glycosidic bond should involve ionization of the ring oxygen or the exocyclic aglycone oxygen (Scheme 1).



Scheme 1. Possible mechanisms for the solvolysis of a glycosidic bond by trifluoroacetolysis.

It is therefore not surprising that a 4-O- or a 2-O-tri-fluoroacetyl group is more effective than a 3-O-trifluoroacetyl group in reducing the rate of solvolysis. Methyl α -D-xylopyranosides with two free hydroxyls were essentially stable under trifluoroacetolysis with TFA/TFAA in the proportions 1:50 but in the proportions 1:1 considerable solvolysis occurred (Table I). Little or no anomerisation was observed except in the case of methyl 3-O-methyl- α -D-xylopyranoside at 1:1 TFA/TFAA.

The partially methylated D-xyloses are rapidly converted

into their pertrifluoroacetylated derivatives. Pertrifluoroacetylated 2,3,4-tri-O-methyl-D-xylose is completely degraded by TFA/TFAA in both the proportions 1:50 and 1:1. This degradation is probably initiated by acid catalyzed elimination reactions. A 4-O-trifluoroacetyl group is more effective in preventing this acid catalyzed degradation than O-trifluoroacetyl groups in the 2- and 3-positions (Table II).

EXPERIMENTAL

Concentrations were performed at reduced pressure with bath temperatures not exceeding 40°C. G.l.c.-m.s. was performed on a combined gas chromatograph-mass spectrometer (Varian MAT 311A). Separations were performed at 170°C on glass capillary columns (50 m x 0.25 mm) wall-coated with SE-30 (LKB-Products, Sweden). Mass spectral data were processed with an on-line computer system (Spectrosystem 100, Varian MAT). Quantitative analyses were performed using the above column fitted in a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionisation detector.

Partially methylated methyl α -D-xylopyranosides were obtained by standard synthetic procedures. The identity and purity of the compounds were established by g.l.c.-m.s. of the corresponding acetates⁸.

Partially methylated D-xyloses were obtained from the corresponding methyl glycoside by hydrolysis with 0.25 M aqueous sulphuric acid at 100°C for 18 h. The identity and purity of the compounds were established by g.l.c.-m.s. of the corresponding alditol acetates.

Trifluoroacetolysis experiments. - The partially methylated methyl α -D-xylopyranoside or partially methylated D-xylose (9 mg) and xylitol or methyl α -D-glucopyranoside (6 mg) (as stable internal standard) were dissolved in methanol (3 ml) and 1 ml of the mixture was analyzed by g.l.c.-m.s. after acetylation (xylosides) and reduction-acetylation (xyloses) to obtain response factors for the quantitative determinations. Other portions (1 ml) were dried and dissolved in TFA/TFAA (1:1 or 1:50, v/v) (4 ml) and were then heated in a sealed glass tube at 100°C for 48 h (Caution! Corrosive mixture under pressure).

The mixtures were then cooled to room temperature and evaporated to dryness. The residues were dissolved in methanol (2 ml) and the solutions were evaporated to dryness. The residues were then dissolved in ethanol:water (2:1, v/v) (2 ml) and reduced with sodium borodeuteride (10 mg). The reduced products were acetylated and analyzed by g.l.c.-m.s. The identity of each component was established by its mass spectrum and retention time on g.l.c. as compared with an authentic sample.

ACKNOWLEDGEMENTS

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III

The Stability of Partially Methylated Methyl α -L-Arabinofuranosides towards Trifluoroacetolysis

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Trifluoroacetolysis is a versatile method for studies on glycoconjugates. Under trifluoroacetolysis conditions that will effect transamidation of 2-acetamido-2-deoxy functions in sugar residues into 2-deoxy-2-trifluoroacetamido groups,¹ most reducing sugars and glycosides are stable and are converted into their pertrifluoroacetylated derivatives.^{2,3} The reason for the stability of glycosides and reducing sugars is that the O-trifluoroacetyl groups which are rapidly introduced exert strong inductive effects which reduce the electron density of the ring and glycosidic oxygens. Using trifluoroacetolysis, methods have been devised for the isolation of N- and O-glycosidically linked carbohydrate chains in glycoproteins,⁴⁻⁶ for specific cleavage of the glycosidic linkage between the oligosaccharide and ceramide portions in glycolipids,⁷ and for specific degradation of reducing 2-acetamido-2-deoxy-hexose residues.^{2,4-6} In order to elucidate the effect of differently positioned O-trifluoroacetyl groups in preventing solvolysis of glycosides, detailed investigations have been carried out using partially methylated methyl α -D-gluco- and xylopyranosides as models.^{8,9} In this communication the model compound studies are extended

Table 1. Trifluoroacetolysis of partially methylated methyl α -L-arabinofuranosides using TFA/TFEA 1:1 and 1:50 at 100°C for 48 h.

Location of methyl group(s)	Starting material 1:1	Starting material 1:50	Recovery (mol%) ^a		Free sugar 1:1	Free sugar 1:50	Total recovery	
			Anomerized product 1:1	Anomerized product 1:50			1:1	1:50
0	68	101	20	<1	14	<1	102	101
2	<1	92	- ^b	6	87	5	87	103
3	20	98	8	<1	64	1	92	99
5	27	97	9	2	59	1	95	100
2,3	-	8	-	3	2	84	2	95
2,5	<1	41	-	<1	6	59	6	100
3,5	5	70	3	6	12	25	20	101
2,3,5	-	-	-	-	-	5	0	5

^a Determined after de-O-trifluoroacetylation, reduction (NaBD₄) and acetylation.

^b - = not detectable.

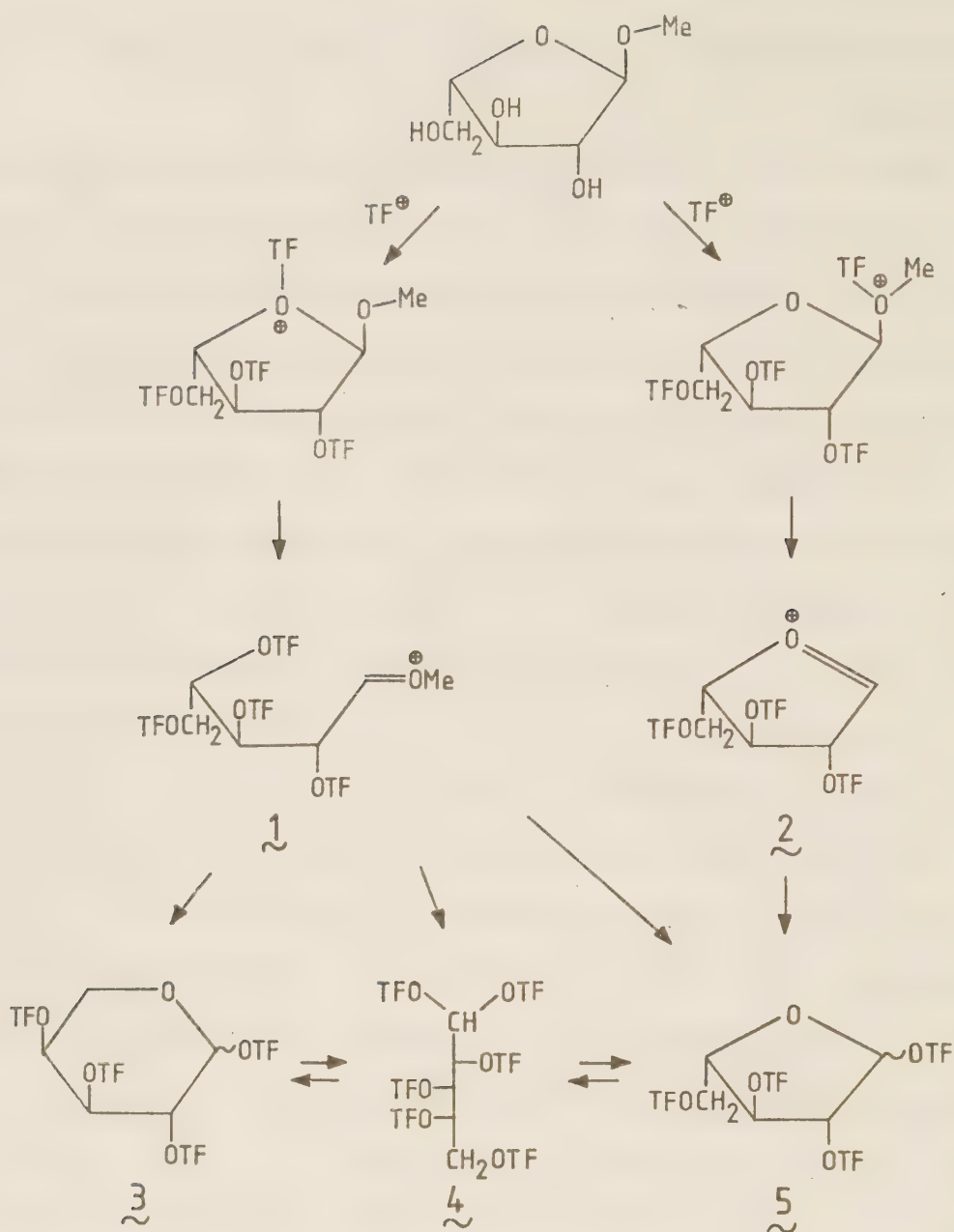
to trifluoroacetolysis of partially methylated methyl α -L-arabinofuranosides.

The partially methylated methyl α -L-arabinofuranosides were synthesized from methyl α -L-arabinofuranoside¹⁰ using conventional techniques. All compounds were obtained as chromatographically pure syrups and their purity and identity was established by GLC-MS of their peracetylated derivatives.

The partially methylated methyl α -L-arabinofuranosides were treated with trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) in the proportions 1:1 and 1:50 at 100°C for 48 h. The reaction mixtures were then de-O-trifluoroacetylated, reduced, acetylated, and analyzed by GLC-MS. The results are summarized in Table 1.

As can be seen from Table 1, methyl α -L-arabinofuranoside is stable as its pertrifluoroacetylated derivative during trifluoroacetolysis using TFA/TFAA 1:50, whereas methyl 2,3,5-tri-O-methyl- α -L-arabinoside is completely solvolysed and further degraded. When the 3- or 5-position is methylated, the glycosidic bond is still essentially stable, but when the hydroxyl group in the 2-position is methylated, there is a slight decrease in stability.

Since solvolysis can be initiated by ionisation of the ring oxygen or the exocyclic glycosidic oxygen (Scheme 1), a 2-O-trifluoroacetyl group is the most effective in retarding this ionisation step since it is situated adjacent to C-1. O-Trifluoroacetyl groups in the 3- or 5-position can only effectively protect the ring oxygen from ionisation. When only one O-trifluoroacetyl group can be introduced the protective action decreases in the order 2->3->5-position. When trifluoro-



Scheme 1

acetolysis was carried out using TFA/TFAA in the proportions 1:1, methyl α -L-arabinofuranoside could only be recovered in about 70% yield. The rest was converted into the β -anomer or the solvolysed product(s). If the 2-position was blocked, the solvolysis was complete, whereas 20-30% of the glycoside was recovered when the 3- or 5-position was blocked. For glycosides

with only one free hydroxyl the 2-hydroxyl compound was the only one that was not completely solvolysed.

The intermediate 1 may rearrange to give the anomerized product (Table 1) and by reacting further it could form the solvolysis products 3, 4 or 5, although none of these products were detected by the analytical method used which involved reduction (NaBD_4) and acetylation. The possible intermediate 2 could only react further to yield the solvolysis product 5 which then could produce 3 and 4. The intermediates 1 and 2 and the pertrifluoroacetylated solvolysis products (3, 4 and 5) may be degraded further via acid catalyzed eliminations. The results obtained in this investigation demonstrate that furanosidic bonds may be stable towards trifluoroacetolysis using TFA/TFAA 1:50 providing that at least two hydroxyl groups are unsubstituted.

Experimental. Concentrations were performed at reduced pressure with bath temperatures not exceeding 40°C . GLC-MS was carried out on a combined gas chromatograph-mass spectrometer (Varian MAT 311A). Separations were performed at 170°C on glass capillary columns (50 m x 0.25 mm) wall-coated with SE-30 (LKB-Products, Sweden). Mass spectral data were processed with an on-line computer system (Spectrosystem 100, Varian MAT). Quantitative analyses were performed using the above column fitted in a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionisation detector.

Partially methylated methyl α -L-arabinofuranosides^{10,11} were obtained by standard synthetic procedures. The identity and purity of the compounds was established by GLC-MS of the

corresponding acetates using principles previously outlined.¹² The identity was further corroborated by GLC-MS¹³ of the alditol acetates after hydrolysis, reduction, and acetylation.

Trifluoroacetolysis experiments. The partially methylated methyl α -L-arabinofuranosides (12 mg) and xylitol or methyl α -D-glucopyranoside (6 mg) (as stable internal standard)³ were dissolved in methanol (3 ml) and 1 ml of the mixture was analyzed by GLC-MS after acetylation to obtain response factors for the quantitative determinations. Other portions (1 ml) were dried and dissolved in TFA/TFAA (1:1 or 1:50, v/v) (4 ml) and were then heated in a sealed glass tube at 100°C for 48 h (Caution! Corrosive mixture under pressure).

The mixtures were then cooled to room temperature and evaporated to dryness. The residues were dissolved in methanol (2 ml) and the solutions were evaporated to dryness. These residues were then dissolved in ethanol:water (2:1, v/v) (2 ml) and reduced with sodium borodeuteride (10 mg). The reduced products were acetylated and analyzed by GLC-MS. The identity of each component was established by its mass spectrum and retention time on GLC as compared with an authentic sample.

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IV

The Effect of Hydroxyl Groups in the Aglycone in the Solvolysis of Glucopyranosides by Trifluoroacetolysis

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Abstract

2,3,4,6-Tetra-O-methyl- β -D-glucosides and 2,3,4-tri-O-methyl- β -D-glucosides having hydroxyl groups in the aglycone moiety have been synthesized. The compounds have been investigated for their stability towards trifluoroacetolysis in order to evaluate the stabilizing effect of hydroxyl groups in the aglycone on solvolysis of the glycosidic bond. It was found that a hydroxyl group in the β - or γ -position to the glycosidic oxygen exerts little effect in retarding the solvolysis of the glycosidic bond. However, two hydroxyl groups in the β -position showed a moderate effect in diminishing the rate of solvolysis.

Introduction

A new reaction, trifluoroacetolysis, has recently been introduced which can be used for structural studies of the carbohydrate portion of glycoconjugates. Methods have been developed for N-deacetylation of glycosidically linked 2-acetamido-2-deoxy-sugars,¹ specific degradation of 2-acetamido-2-deoxy-

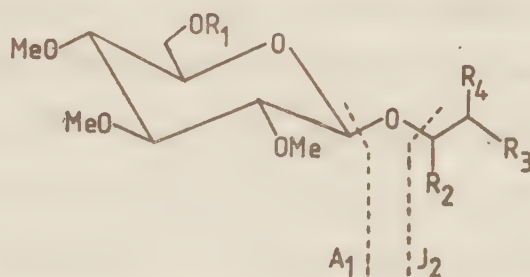
sugars at the reducing end of oligosaccharides,²⁻⁵ isolation of N- and O-glycosidically linked carbohydrate chains in glycoproteins,³⁻⁵ and specific cleavage of the glycosidic bond to ceramide in glycolipids.⁶ These methods all feasible because the hydroxyl groups of carbohydrates are rapidly trifluoroacetylated during trifluoroacetolysis and the electron-withdrawing effect of the O-trifluoroacetyl groups prevents solvolysis of glycosides⁷ and acid catalyzed degradation of reducing sugars.² In recent studies^{8,9} on trifluoroacetolysis of partially methylated methyl α -D-glucopyranosides and methyl α -D-xylopyranosides, the effect of O-trifluoroacetyl groups in various positions in inhibition of solvolysis was evaluated. We now report a study on the stabilizing effects of hydroxyl groups in the aglycone part of oligosaccharides with the aid of synthesized model compounds.

RESULTS AND DISCUSSION

The compounds 2-hydroxyethyl 2,3,4,6-tetra-O-methyl- β -D-glucoside (1), 1-O-(2,3,4,6-tetra-O-methyl- β -D-glucosyl)-D,L-glycerol (2), 2-O-(2,3,4,6-tetra-O-methyl- β -D-glucosyl)-glycerol (3), 1-O-(2,3,4,6-tetra-O-methyl- β -D-glucosyl)-2-O-methyl-D,L-glycerol (4), 2-hydroxyethyl 2,3,4-tri-O-methyl- β -D-glucoside (5), and 2-O-(2,3,4-tri-O-methyl- β -D-glucosyl)-glycerol (6) have been synthesized using conventional techniques. Compounds 2 and 4 were synthesized by coupling 2,3,4,6-tetra-O-acetyl- α -D-glucosyl bromide to D,L-1,2-O-isopropylidene-glycerol yielding 1-O-(2,3,4,6-tetra-O-acetyl- β -D-glucosyl)-

2,3-O-isopropylidene-D,L-glycerol as an intermediate. The two diastereoisomers of this compound were not separated, and thus compounds 2 and 4 were obtained as diastereoisomeric mixtures.

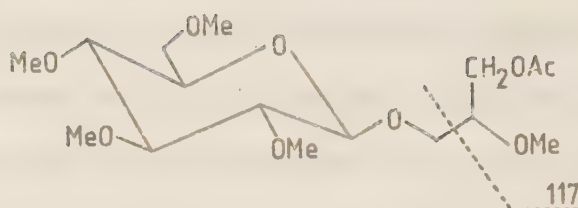
Mass spectrometry of compounds 1-6 as their peracetylated derivatives. The mass spectra of the peracetylated compounds 1-6 all show strong fragments in the J-series¹⁰ which allow calculation of the molecular weight of the aglycone units (Scheme 1). Thus peracetylated compounds 1 and 5 give ions with m/e 147 (J_1) and m/e 87 (J_2), compounds 2, 3, and 6 ions with m/e 219 (J_1) and m/e 159 (J_2), and compound 4 ions with m/e 191 (J_1) and m/e 131 (J_2).



		R ₁	R ₂	R ₃	R ₄
peracetylated	<u>1</u>	Me	H	H	OAc
"	<u>2</u>	Me	H	OAc	CH ₂ OAc
"	<u>3</u>	Me	CH ₂ OAc	H	OAc
"	<u>4</u>	Me	H	OMe	CH ₂ OAc
"	<u>5</u>	Ac	H	H	OAc
"	<u>6</u>	Ac	CH ₂ OAc	H	OAc

Scheme 1

The aglycone unit is further characterized, in peracetylated compound 4, by a fragment at m/e 117 obtained by α -cleavage at the methoxyl group (Scheme 2).



Scheme 2

A weak but recognizable A-series¹⁰ of fragments (Scheme 1) gives ions at m/e 187 (A_2) and m/e 155 (A_3) for peracetylated 1-4 and at m/e 215 (A_2) and m/e 155 (A_3) for peracetylated 5 and 6.

Trifluoroacetolysis of compounds 1-6 and the corresponding methyl β -D-glucosides. These compounds together with stable internal standards were treated with trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) in the proportions 1:1, 1:4, and 1:50 (v/v) at various temperatures for 48 h. Each reaction mixture was de-O-trifluoroacetylated, reduced, acetylated, and analyzed qualitatively and quantitatively by GC-MS. The results are shown in Tables 1 and 2.

Methyl 2,3,4,6-tetra-O-methyl- β -D-glucoside and the compounds 1-4 were completely solvolyzed with TFA/TFAA in the proportions 1:1 and 1:50 at 100°C for 48 h. The trifluoroacetylated 2,3,4,6-tetra-O-methyl-D-glucose formed by the solvolysis reaction was then further destroyed by acid catalyzed elimination reactions. At 60°C (Table 1), however, about half of the amount of methyl 2,3,4,6-tetra-O-methyl- β -D-glucoside could be recovered after trifluoroacetolysis using TFA/TFAA in the proportions 1:50. Compound 1 which has a hydroxyl group in the glycone in β -position to the exocyclic glycosidic oxygen

Table 1. Trifluoroacetolysis of methyl 2,3,4,6-tetra-O-methyl- β -D-glucoside (Me 2,3,4,6-OMe-Glc) and compounds 1-4 in TFA/TFAA (1:1 and 1:50) at 60°C for 48 h.

Compound	Recovery (mol%) ^a	
	1:1	1:50
Me 2,3,4,6-OMe-Glc	6	46
1	4	77
2	22	85
3	30	93
4	5	74

^a Determined after de-O-trifluoroacetylation, reduction (NaBD₄) and acetylation.

Table 2. Trifluoroacetolysis of methyl 2,3,4-tri-O-methyl- β -D-glucoside (Me 2,3,4-OMe-Glc) and compounds 5 and 6 in TFA/TFAA (1:1, 1:4, and 1:50) at 100°C for 48 h.

Compound	Recovery (mol%) ^a		
	1:1	1:4	1:50
Me 2,3,4-OMe-Glc	-	4 (20) ^b	47 (36)
5	-	6	59
6	1	24	80

^a Determined after de-O-trifluoroacetylation, reduction (NaBD₄), and acetylation.

^b Values in parenthesis represent anomerized product.

was more stable and could be recovered in 77% yield after trifluoroacetolysis in TFA/TFAA 1:50. Similar results were obtained for compound 4 which has a hydroxyl group in the γ -position. With hydroxyl groups in the β - and γ -position (2)

the stability was somewhat greater and with two hydroxyl groups in the β -position (3) almost complete stability in TFA/TFAA 1:50 was observed. In TFA/TFAA 1:1 the compounds 2 and 3 were noticeably more stable than compounds 1, 4, and methyl 2,3,4,6-tetra-O-methyl- β -D-glucoside.

Since solvolysis by TFA/TFAA is initiated by oxonium ion formation of either the ring oxygen or the exocyclic glycosidic oxygen and since in compounds 1-4 no electron-withdrawing O-trifluoroacetyl group is protecting the ring oxygen from forming such an ion, the small stabilizing effect exerted by the O-trifluoroacetyl groups in the aglycone could be due to preferential oxonium ion formation of the ring oxygen.

In order to investigate if the protective action of O-trifluoroacetyl groups in the aglycone was larger if ionization of the ring oxygen was hindered, the compounds 5 and 6 with a 6-hydroxyl group were prepared and their solvolysis in TFA/TFAA was compared with methyl 2,3,4-tri-O-methyl- β -D-glucoside. The results (Table 2) show that one or two hydroxyl groups in the β -position prevented solvolysis to about the same extent as the hydroxyl groups in the model compounds 1 and 3 as compared with the corresponding methyl β -D-glucosides. The results presented in this study show that hydroxyl groups in the aglycone have an effect in preventing solvolysis of glycosides in TFA/TFAA. However, the effect was small. The effect should nevertheless be important for stabilizing labile sugar residues in oligo- and polysaccharides especially when 3-linked to another sugar unit.

EXPERIMENTAL

General methods. GLC was carried out on a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionization detector. Separations were performed on an SE-30 W.C.O.T. glass capillary column (25 m x 0.25 mm) at 200°C. GLC-MS was performed on a Varian MAT 311A instrument fitted with the above column. The spectra were recorded at 70 eV with an ionization current of 3 mA and an ion source temperature of 120°C. The spectra were processed on an on-line computer system (Spectrosystem 100, Varian MAT). Silica gel column chromatography was carried out using the following solvent systems: a) ethyl acetate:petroleum ether, 1:1 (v/v); b) ethyl acetate containing 3% methanol; c) ethyl acetate containing 8% methanol; d) ethyl acetate containing 15% methanol; e) ethyl acetate; and f) ethyl acetate:petroleum ether, 2:3 (v/v). Reactions and separations were monitored by thin layer chromatography (TLC) on silica gel plates.

2-Hydroxyethyl 2,3,4,6-tetra-O-methyl-β-D-glucoside (1). 1 was obtained from 2-hydroxyethyl 2,3,4,6-tetra-O-acetyl-β-D-glucoside^{11,12} by a) tritylation, b) de-O-acetylation, c) methylation, and d) detritylation.

a) Tritylation. 2-Hydroxyethyl 2,3,4,6-tetra-O-acetyl-β-D-glucoside (1.6 g) and chlorotriphenylmethane (1.6 g) were added to 20 ml of pyridine and the mixture was left at room temperature for 48 h. The reaction mixture was thereafter partitioned between water (50 ml) and chloroform (50 ml). The chloroform layer was washed with ice-cold aqueous potassium hydrogen sulphate, aqueous sodium carbonate, and

water. After drying the chloroform layer over anhydrous sodium sulphate it was evaporated to dryness. The residue was crystallized from absolute ethanol. The crude product (2.1 g) was used in the next step without further purification.

b) De-O-acetylation. 2-O-Trityloxyethyl 2,3,4,6-tetra-O-acetyl- β -D-glucoside (1.2 g) (above) was dissolved in M ammonia in methanol/water (4:1, v/v, 50 ml). After 24 h at room temperature the reaction mixture was evaporated to dryness. The yield of crude amorphous O-tritylethyl β -D-glucopyranoside was 0.9 g. The product was homogeneous on TLC (solvent e).

c) Methylation. 2-O-Trityloxyethyl β -D-glucopyranoside (above) (600 mg) was methylated with sodium methylsulphinylmethanide/methyl iodide in dimethylsulphoxide according to the procedure of Hakomori.¹³ The methylated product was purified by chromatography on a silica gel column eluted with solvent a. The yield of purified 2-O-trityloxyethyl 2,3,4,6-tetra-O-methyl- β -D-glucoside was 620 mg.

d) Detritylation. 2-O-Trityloxyethyl 2,3,4,6-tetra-O-methyl- β -D-glucoside (600 mg) (above) was detritylated by treatment with 0.2 M hydrochloric acid in methanol (50 ml) for 18 h. The reaction mixture was neutralized (Ag_2CO_3), filtered, and evaporated to dryness. The crude product was purified twice by chromatography on a silica gel column eluted with solvent b. The yield of 1 as a chromatographically pure syrup was 240 mg. Part of 1 was acetylated and analyzed by GLC-MS. It gave a single peak with a mass spectrum containing ions at m/e 43 (60), 45 (42), 71 (22),

73 (18), 75 (15), 87 (88), 88 (100), 89 (11), 101 (68), 102 (7), 111 (3), 127 (4), 147 (22), 155 (2), 187 (4), and 221 (2).

1-O-(2,3,4,6-Tetra-O-methyl-β-D-glucosyl)-D,L-glycerol (2).

2 was obtained from 1-O-(2,3,4,6-tetra-O-acetyl-β-D-glucosyl)-2,3-O-isopropylidene-D,L-glycerol^{12,14} by a) de-O-acetylation, b) methylation, and c) removal of the isopropylidene group.

a) De-O-acetylation. 1-O-(2,3,4,6-tetra-O-acetyl-β-D-glucosyl)-2,3-O-isopropylidene-D,L-glycerol (1.8 g) was de-O-acetylated as previously described in the preparation of 1. The yield of crude 1-O-(β-D-glucopyranosyl)-2,3-O-isopropylidene-D,L-glycerol was 1.2 g. The product was homogenous on TLC (solvent e).

b) Methylation. 1-O-(β-D-Glucopyranosyl)-2,3-O-isopropylidene-D,L-glycerol (1.0 g) was methylated as previously described.¹³ The crude product was purified twice by chromatography on a silica gel column eluted with solvent e. The yield of chromatographically pure 1-O-(2,3,4,6-tetra-O-methyl-β-D-glucosyl)-2,3-O-isopropylidene-D,L-glycerol was 0.9 g. A single spot was seen on TLC (solvent e).

c) Removal of the isopropylidene group. 1-O-(2,3,4,6-tetra-O-methyl-β-D-glucosyl)-2,3-O-isopropylidene-D,L-glycerol (800 mg) (above) was treated with 0.5 M hydrochloric acid in methanol (40 ml) at room temperature for 24 h. After this time no starting material remained and a new single spot was seen on TLC (solvent c). The reaction mixture was neutralized with silver carbonate, filtered,

and evaporated to dryness. The crude product was purified by chromatography on a silica gel column eluted with solvent c. The chromatographically pure product was obtained as a syrup. The yield of 2 was 610 mg. Part of 2 was acetylated and analyzed by GLC-MS. This analysis showed two peaks in the ratio 2:3. Both components gave the same mass spectrum with ions at m/e 43 (52), 45 (22), 71 (10), 73 (7), 75 (12), 88 (100), 89 (9), 101 (52), 102 (8), 111 (2), 127 (3), 145 (3), 159 (50), 187 (1), and 219 (7). The appearance of two peaks after GLC-MS is expected since it should be a diastereoisomeric mixture. In order to exclude the possibility of an anomeric mixture, 2 was analyzed by ¹H NMR. This spectrum showed only one doublet (δ , 4.34; J, 10 Hz) in the region of anomeric protons demonstrating that 2 contains only β -anomers.

2-O-(2,3,4,6-tetra-O-methyl- β -D-glucosyl)-glycerol (3).

3 was obtained from 2-O-(2,3,4,6-tetra-O-acetyl- β -D-glucosyl)-1,3-O-benzylidene-glycerol^{12,15} by a) de-O-acetylation, b) methylation, and c) removal of the O-benzylidene group.

a) De-O-acetylation. 2-O-(2,3,4,6-tetra-O-acetyl- β -D-glucosyl)-1,3-O-benzylidene-glycerol (1.6 g) was de-O-acetylated as previously described in the preparation of 1 and 2. The yield of crude 2-O-(β -D-glucopyranosyl)-1,3-O-benzylidene-glycerol was 1.1 g. The product was homogeneous on TLC (solvent e).

b) Methylation. 2-O-(β -D-glucopyranosyl)-1,3-O-benzylidene-glycerol (600 mg) was methylated as previously described¹³. The crude product was purified twice by chromatography on

a silica gel column eluted with solvent e. The yield of a chromatographically pure syrup was 650 mg.

c) Removal of the O-benzylidene group. 2-O-(2,3,4,6-tetra-O-methyl- β -D-glucosyl)-1,3-O-benzylidene-glycerol (400 mg) (above) was treated at room temperature with 0.5 M hydrochloric acid in methanol (40 ml) for 24 h. After neutralization (Ag_2CO_3) and filtration the reaction mixture was evaporated to dryness. The crude product was purified by chromatography on a silica gel column eluted with solvent c. The yield of chromatographically pure 3 was 260 mg. Part of 3 was acetylated and analyzed by GLC-MS. This analysis showed a single peak with a mass spectrum containing the ions m/e 43 (71), 45 (28), 71 (13), 73 (9), 75 (12), 88 (100), 89 (7), 101 (48), 111 (4), 155 (2), 159 (43), 160 (3), 187 (5), 219 (15).

1-O-(2,3,4,6-tetra-O-methyl- β -D-glucosyl)-2-O-methyl-D,L-glycerol (4). 4 was obtained from 2 by a) tritylation, b) methylation, and c) detritylation.

a) Tritylation. 2 (200 mg) and chlorotriphenylmethane (300 mg) were added to pyridine (5 ml). The mixture was stirred at room temperature for 48 h. After this time practically no starting material remained and a new major compound had been formed as seen by TLC (solvent e). The reaction mixture was worked up as described above (under synthesis of 1). The crude product was purified by chromatography on a silica gel column eluted with solvent e. Pure 1-O-(2,3,4,6-tetra-O-methyl- β -D-glucosyl)-3-O-trityl-D,L-glycerol was obtained as a syrup (330 mg).

b) Methylation. 1-O-(2,3,4,6-tetra-O-methyl-β-D-glucosyl)-3-O-trityl-D,L-glycerol (300 mg) (above) was methylated as previously described.¹³ The crude product was purified twice by chromatography on a silica gel column eluted with solvent e. Chromatographically pure 1-O-(2,3,4,6-tetra-O-methyl-β-D-glucosyl)-2-O-methyl-3-O-trityl-D,L-glycerol was obtained as a syrup (240 mg).

c) Detritylation. 1-O-(2,3,4,6-tetra-O-methyl-β-D-glucosyl)-2-O-methyl-3-O-trityl-D,L-glycerol (200 mg) was detritylated by treatment with 0.2 M hydrochloric acid in methanol (40 ml) as described above (under synthesis of 1). The crude product was purified by chromatography on a silica gel column eluted with solvent b to yield 4 (100 mg) as a pure syrup. Part of 4 was acetylated and analyzed by GLC-MS giving a single peak with the ions m/e 43 (26), 45 (28), 71 (37), 73 (11), 75 (16), 88 (100), 89 (11), 101 (68), 102 (7), 111 (6), 117 (8), 127 (5), 131 (95), 132 (6), 145 (3), 155 (2), 187 (8), 191 (27), 192 (2).

2-Hydroxyethyl 2,3,4-tri-O-methyl-β-D-glucoside (5). 5 was obtained from 2-O-trityloxyethyl β-D-glucopyranoside (see under preparation of 1) by a) tritylation, b) methylation, and c) detritylation.

a) Tritylation. 2-O-Trityloxyethyl β-D-glucopyranoside (250 mg) and chlorotriphenylmethane (150 mg) were added to pyridine (5 ml) and the mixture was stirred for 48 h at room temperature. After this time the reaction mixture was kept at 100°C for 1 h. After cooling the reaction mixture was worked up as previously described. The crude

product (2-O-trityloxyethyl 6-O-trityl- β -D-glucopyranoside) was not further purified.

b) Methylation. The crude 2-O-trityloxyethyl 6-O-trityl- β -D-glucopyranoside (above) was methylated as previously described.¹² The crude product (2-O-trityloxyethyl 2,3,4-tri-O-methyl-6-O-trityl- β -D-glucoside) was not isolated, but the residue after the work-up was used directly in the next step.

c) Detritylation. The crude 2-O-trityloxyethyl 2,3,4-tri-O-methyl-6-O-trityl- β -D-glucoside (above) was treated with 0.2 M hydrochloric acid in methanol/ethanol (1:1, v/v) for 18 h at room temperature. After neutralization (Ag_2CO_3) and filtration the reaction mixture was evaporated to dryness. The crude product was purified on a silica gel column eluted with solvent c. The main component consisting of 5 was further purified as its peracetylated derivative on a silica gel column eluted with solvent f. The peracetylated 5 was obtained as a chromatographically pure syrup (150 mg). Analysis of peracetylated 5, by GLC-MS, showed a single peak with a mass spectrum containing the ions m/e 43 (23), 45 (6), 73 (6), 75 (4), 87 (43), 88 (100), 89 (3), 101 (44), 147 (9), 155 (4), 215 (2).

The pure peracetylated 5 (140 mg) was de-O-acetylated with M ammonia in methanol:water (4:1) (20 ml) as described above (see under synthesis of 1). The crude product was purified on a silica gel column, eluted with solvent c, to yield chromatographically pure, amorphous 5 (90 mg).

2-O-(2,3,4-tri-O-methyl-β-D-glucosyl)-glycerol (6).

6 was obtained from 2-O-(β-D-glucopyranosyl)-1,3-O-benzylidene-glycerol (see under preparation of 3) by a) tritylation, b) methylation, and c) removal of O-trityl and O-benzylidene groups.

a) Tritylation. 2-O-(β-D-glucopyranosyl)-1,3-O-benzylidene-glycerol (250 mg) and chlorotriphenylmethane (220 mg) were added to pyridine (5 ml) and the mixture was stirred for 48 h at room temperature. After this time the reaction mixture was heated for 1 h at 100°C. After cooling the reaction mixture was worked up as previously described. The crude product 2-O-(6-O-trityl-β-D-glucopyranosyl)-1,3-O-benzylidene-glycerol was not purified further.

b) Methylation. The crude 2-O-(6-O-trityl-β-D-glucopyranosyl)-1,3-O-benzylidene-glycerol (above) was methylated as previously described. The crude product 2-O-(2,3,4-tri-O-methyl-6-O-trityl-β-D-glucosyl)-1,3-O-benzylidene-glycerol was not isolated, but the residue after the work-up was used directly in the next step.

c) Removal of O-trityl and O-benzylidene groups. The crude 2-O-(2,3,4-tri-O-methyl-6-O-trityl-β-D-glucosyl)-1,3-O-benzylidene-glycerol was treated with 0.5 M hydrochloric acid in methanol for 18 h at room temperature. After neutralization (Ag₂CO₃) and filtration the reaction mixture was evaporated to dryness. The crude product was purified on a silica gel column eluted with solvent d. The main component consisting of 6 was further purified as its peracetylated derivative on a silica gel column eluted with solvent a. The peracetylated 6 was obtained as a

chromatographically pure syrup (250 mg). Analysis of peracetylated 6, by GLC-MS, showed a single peak with a mass spectrum containing the ions m/e 42 (13), 43 (19), 71 (10), 73 (6), 75 (5), 87 (8), 88 (100), 101 (61), 127 (6), 155 (8), 159 (73), 160 (9), 183 (3), 215 (3), 219 (20). The pure peracetylated 6 (240 mg) was de-O-acetylated with M ammonia in methanol:water (4:1) (20 ml) as described above (see under synthesis of 1). The crude product was purified on a silica gel column eluted with solvent d, to yield chromatographically pure 6 as a syrup (150 mg).

Trifluoroacetylolysis experiments. Each of the compounds 1-6, methyl 2,3,4,6-tetra-O-methyl- β -D-glucoside¹⁶, and methyl 2,3,4-tri-O-methyl- β -D-glucoside¹⁶ (25 mg) were dissolved together with methyl α -D-glucopyranoside (15 mg) (as stable internal standard)⁷ in methanol (5 ml). Part of the mixture (1 ml) was acetylated and analyzed by GLC-MS after acetylation to obtain response factors for quantitative determinations. Other portions (1 ml) were dried and dissolved in TFA/TFAA (1:1, 1:4, and 1:50, v/v) (4 ml) and then heated in a sealed glass tube at 60°C or 100°C for 48 h. (Caution! Corrosive mixture under pressure.)

The mixtures were then cooled to room temperature and evaporated to dryness. The residues were dissolved in methanol (2 ml) and the solutions were evaporated to dryness. The residues were then dissolved in ethanol:water (2:1, v/v) (2 ml) and reduced with sodium borohydride (10 mg) and acetylated. The components in the product were identified by GLC-MS and compared with authentic materials.

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Structural Studies on the Carbohydrate Portion of Human Anti-thrombin III

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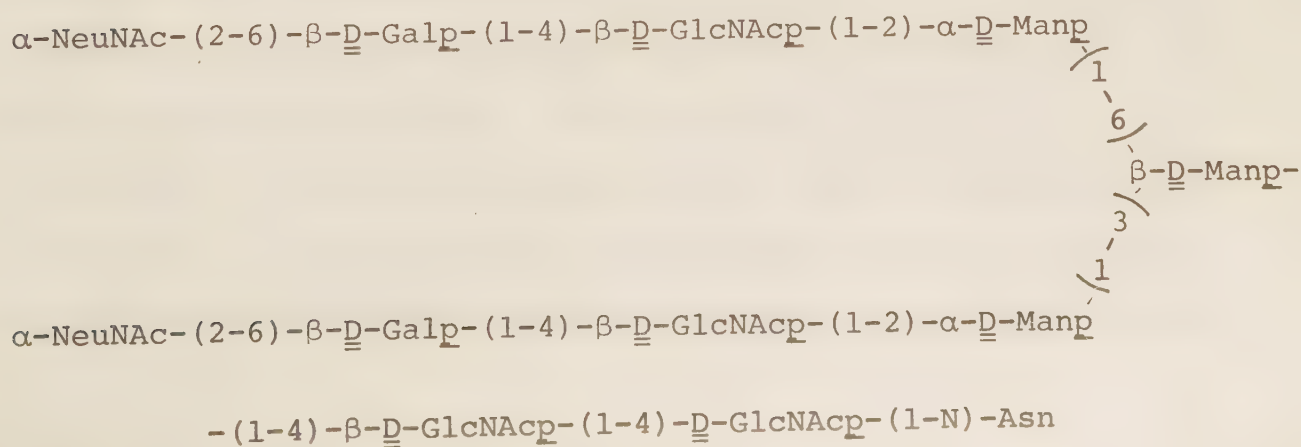
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Summary

Human antithrombin III has been shown to contain four identical N-glycosidically linked carbohydrate chains per molecule. These carbohydrate chains have been investigated by sugar and methylation analysis before and after removal of N-acetylneuraminic acid residues. The chains have been further investigated by Smith degradation, trifluoroacetylytic degradation and degradation after chromium trioxide oxidation. As a result of these studies the following structure is proposed for the carbohydrate chains in human antithrombin III:



Introduction

Antithrombin III is a slightly acidic single-chain glycoprotein containing 10-15% of carbohydrates (1-3). It has a molecular weight of about 60,000 (1-3). Antithrombin III is an important regulating factor of blood coagulation and inhibits thrombin as well as several of the other serine proteases that participate in the coagulation process (4). The polysaccharide heparin binds tightly to antithrombin III and the inhibition of thrombin by antithrombin III is strongly accelerated by the addition of this polysaccharide (4,5). Antithrombin III can be purified from plasma by various methods, including affinity chromatography on heparin-agarose (3). The present communication deals with structural studies on the carbohydrate portion of human antithrombin III.

MATERIALS AND METHODS

Analytical Methods - Colorimetric methods were used for the determination of total hexose (6), total hexosamine (7), and N-acetylneuraminic acid (8). Sodium ion was determined by flame photometry. Sugar analysis was performed after hydrolysis with 90% aqueous formic acid at 100°C for 5 h followed by hydrolysis with 0.25 M aqueous sulphuric acid at 100°C for 18 h, by GLC (9) and MS (10). Methylation analysis was performed as previously described (11). GLC was carried out on a Perkin-Elmer 3950 gas chromatograph equipped with a flame ionisation detector. Separations were performed on (a) an SE-30 W.C.O.T. glass capillary column (50 m x 0.25 mm) at 190°C

(for partially methylated alditol acetates) and at 180^o-330^oC (for permethylated oligosaccharide alditols), (b) a glass column (2 m x 0.5 cm) packed with 3% ECNSS-M on Chromosorb Q at 200^oC (for alditol acetates). GLC-MS was performed on a Varian MAT 311A instrument fitted with the appropriate column. The spectra were recorded at 70 eV, with an ionisation current of 3 mA and an ion source temperature of 120^oC. The spectra were processed on an on-line computer system (Spectro-system 100, Varian MAT).

Human Antithrombin III - This was obtained from AB Kabi (Stockholm, Sweden) (3). The preparation was homogeneous on gel filtration and electrophoresis.

Preparation of Asialoantithrombin III - This was obtained by treatment of antithrombin III with Vibrio cholerae neuraminidase (Behringwerke AG, Marburg/Lahn, West Germany) or by mild acid hydrolysis (12). Both procedures removed more than 95% of the NeuNAc residues.

Trifluoroacetolysis of Antithrombin III - Freeze-dried antithrombin III (100 mg) was added to a solution of TFA/TFAA (50 ml, 1:1, v/v) and the reaction mixture was sealed in a glass vessel. The reaction mixture was heated at 100^oC for 48 h (Caution! Corrosive mixture under pressure!). The resulting clear, dark solution was cooled and evaporated to dryness. The product was dissolved in methanol (25 ml) and the solution was evaporated to dryness. The residue was then treated at room temperature with 50% aqueous acetic acid (25 ml) and after 30 min at 100^oC the reaction mixture was evaporated to dryness. The product was partitioned between diethyl ether (50 ml) and

water (25 ml). The ethereal layer was extracted twice with water (25 ml). The combined water phases were extracted once with diethyl ether (25 ml) and concentrated to 10 ml. To the water solution was added sodium borodeuteride (100 mg) and after 18 h the reduction mixture was acidified with glacial acetic acid and concentrated to dryness. Boric acid was removed by three codistillations with methanol (10 ml) and the resulting product was desalted on a Sephadex G-25 column (3 x 40 cm) eluted with distilled water. The desalted product was acetylated with acetic anhydride/pyridine (1:1, v/v, 10 ml) at 100°C for 1 h. After evaporating the reagents the acetylated product was de-O-acetylated by treatment with 1 M ammonia in 75% aqueous methanol (25 ml) at room temperature for 18 h. The reaction mixture was evaporated to dryness and fractionated on a Sephadex G-25 column (1.6 x 40 cm) eluted with distilled water. One carbohydrate-containing peak was obtained which was pooled and lyophilized to yield 4 mg of product.

Chromium Trioxide Oxidation of the Oligosaccharide Obtained after Trifluoroacetolysis of Antithrombin III - To about 3 mg of acetylated oligosaccharide obtained by trifluoroacetolysis of antithrombin III (see above) was added xylitol pentaacetate as internal standard and the mixture was divided into two portions. One portion was subjected to sugar analysis and the other portion was dissolved in glacial acetic acid (2 ml). To the acetic acid solution was added powdered chromium trioxide (20 mg) and the resulting suspension was agitated in an ultrasonic bath at 50°C for 2 h. The suspension was then cooled, diluted with water (50 ml) and the resulting solution was extracted three times with chloroform (10 ml). The combined

chloroform phases were washed with water (2 x 10 ml) and evaporated to dryness. The oxidized product was subjected to sugar analysis and investigated by GLC-MS after permethylation (13,14).

Smith Degradation - Trifluoroacetolysis of Antithrombin III

- Antithrombin III (100 mg) was dissolved in sodium acetate buffer (0.1 M, pH 4.0) (50 ml) and to this solution was added 170 mg of sodium metaperiodate dissolved in sodium acetate buffer (0.1 M, pH 4.0) (50 ml). The periodate oxidation was carried out at 4°C in the dark. After 48 h excess of periodate was destroyed by the addition of ethylene glycol (1 ml). Sodium borohydride 250 mg was added portion-wise and the reduction was carried out at 4°C for 20 h. The reduction mixture was acidified (pH 3) by the addition of glacial acetic acid. The solution was concentrated to dryness and boric acid was removed by codistillation with methanol (3 x 10 ml). The resulting product was desalted by dialysis. The yield of periodate-oxidized and reduced material was 80 mg. Part of this material was subjected to sugar and methylation analysis showing -3)-[-6]-D-Manp-(1- and -4)-D-GlcNAcp-(1- in the relative molar proportions 1:4.

Periodate-oxidized and reduced material (70 mg) was hydrolysed in 0.1 M aqueous hydrochloric acid (40 ml) for 1 h at 80°C. The hydrolysis mixture was cooled, neutralised with sodium hydrogen carbonate and dialyzed extensively against distilled water. The dialyzed material was lyophilized and subjected to sugar and methylation analysis. The dialysis water was concentrated, reduced with sodium borodeuteride and acetylated. The acetylated product was partitioned between chloroform and water and the chloroform phase was washed with water. The acetylated product was then permethylated and analyzed by

GLC-MS. A single oligosaccharide peak was obtained with a mass spectrum and GLC-retention time identical to that of β -D-GlcNAc β -(1-2)-[1-D]-glycerol (14).

Trifluoroacetolysis of Asialoantithrombin III - Lyophilized asialoantithrombin III (180 mg) was trifluoroacetolysed in TFA/TFAA (100 ml, 1:100, v/v) for 48 h at 100°C. The reaction mixture was worked up as described above for trifluoroacetolysis of antithrombin III except that no de-O-acetylation was carried out on the acetylated product. The yield of acetylated oligosaccharide was 30 mg.

Chromium Trioxide Oxidation of the Acetylated Oligosaccharide Obtained after Trifluoroacetolysis of Asialoantithrombin III - Part of the acetylated oligosaccharide (10 mg), obtained by trifluoroacetolytic degradation of asialoantithrombin III, was subjected to chromium trioxide oxidation using the procedure described above.

RESULTS AND DISCUSSION

Sugar analysis of human antithrombin III showed D-Man (3.7%), D-Gal (2.4%), D-GlcNAc (5.5%), and NeuNAc (3.9%) corresponding to the relative molar proportions 3.1:2.0:3.7:1.9. Methylation analysis of antithrombin III before and after desialylation (Table I) demonstrated the following structural elements in the carbohydrate portion:

NeuNAc-(2-6)- <u>D</u> -Gal β -(1-	2 residues
-4)- <u>D</u> -GlcNAc β -(1-	4 residues
-2)- <u>D</u> -Man β -(1-	2 residues
-3)- <u>D</u> -Man β -(1-	1 residue
6	

TABLE I

Methyl ethers obtained from hydrolysates of methylated antithrombin III
and desialylated antithrombin III

Sugars	T-value ^a SE-30	Relative molar proportions ^b Antithrombin Antithrombin
2,3,4,6-O-Me-D-Gal	1.07	<0.05 (0) ^c 2.1 (2)
2,3,4-O-Me-D-Gal	1.82	1.9 (2) <0.05 (0)
3,4,6-O-Me-D-Man	1.45	2.1 (2) 2.0 (2)
3,6-O-Me-D-Man	2.40	1.0 (1) 1.0 (1)
3,6-O-Me-D-GlcN(Me)Ac	4.15	+ (4) ^d + (4) ^d
3,6-O-Me-D-GlcNAC	4.32	

^a Retention times of the corresponding alditol acetates relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

^b Desialylated.

^c Figures in parentheses represent nearest integer.

^d The relative molar proportions were calculated from the sugar analysis (see text).

In order to determine the sequential arrangement and anomeric configurations of the sugar units in antithrombin III it was subjected to trifluoroacetylolytic degradation (15-20) at conditions (TFA/TFAA, 1:1, 100°C, 48 h) that will cleave off the carbohydrate chains from the protein moiety, cleave off 6-linked NeuNAc residues, degrade reducing D-GlcNAc residues and transamidate D-GlcNAc units into D-GlcNTF residues. The trifluoroacetolysis also degraded the protein part by transamidation. After de-O- and de-N-trifluoroacetylation by reduction with sodium borodeuteride followed by N-acetylation an oligosaccharide was obtained. Sugar analysis showed D-Gal, D-Man, D-GlcNAc and D-[1-D]-Man-OL in the relative proportions 2.0:1.9:2.1:1.0 respectively. Methylation analysis gave 2,3,4,6-tetra-O-methyl-D-Gal, 3,4,6-tri-O-methyl-D-Man, 3,6-di-O-methyl-D-GlcNAc (and the N-methylated derivative) and 1,2,4,5-tetra-O-methyl-D-[1-D]-Man-OL in the relative molar proportions 2.0:2.2:2.1:0.9 respectively. The oligosaccharide was acetylated and subjected to chromium trioxide oxidation in acetic acid (21). Chromium trioxide in acetic acid will oxidize glycosides, with the aglycone equatorially oriented (β -linked), into 5-hexulosonates whereas glycosides with the aglycone axially oriented (α -linked) are unaffected. Sugar analysis of the acetylated, oxidized oligosaccharide showed D-Man and D-[1-D]-Man-OL in the relative molar proportions 2:1 together with only traces of D-Gal and D-GlcNAc demonstrating that the D-Gal and D-GlcNAc residues were β -linked. On treatment of the acetylated, oxidized oligosaccharide with base the 5-hexulosonate linkages are broken liberating the aglycones. Permethylation of the base-treated product and analysis with GLC-MS showed a single compo-

with retention time and mass spectrum identical to β - $\underline{\underline{D}}$ -GlcNAc $\underline{\underline{p}}$ -(1-2)-[1-D]-glycerol. The nondialyzable fraction gave on sugar analysis only $\underline{\underline{D}}$ -Man and $\underline{\underline{D}}$ -GlcNAc in the relative molar proportions 1:2 and methylation analysis showed that the $\underline{\underline{D}}$ -Man residue was terminal and the $\underline{\underline{D}}$ -GlcNAc residues were substituted in the 1- and 4-positions. The demonstration of β - $\underline{\underline{D}}$ -GlcNAc $\underline{\underline{p}}$ -(1-2)-[1-D]-glycerol in the dialyzable fraction shows that the oligosaccharide obtained by trifluoroacetolytic degradation of antithrombin III using TFA/TFAA, 1:1, must have the structure 1.

In order to determine the anomeric configuration of the branched $\underline{\underline{D}}$ -Man residue and the $\underline{\underline{D}}$ -GlcNAc residue substituted with the branched $\underline{\underline{D}}$ -Man unit the acetylated oligosaccharide alditol obtained by trifluoroacetolytic degradation, reduction (NaBH_4) and acetylation of asialoantithrombin III was subjected to chromium trioxide oxidation. The acetylated oligosaccharide alditol gave on sugar analysis $\underline{\underline{D}}$ -Gal, $\underline{\underline{D}}$ -GlcNAc, $\underline{\underline{D}}$ -Man and $\underline{\underline{D}}$ -GlcNAc-OL in the molar proportions 2:3:3:1 demonstrating that trifluoroacetolytic degradation of asialoantithrombin III using TFA/TFAA, 1:100, results in degradation of the protein part and cleavage of the amide bond between the innermost $\underline{\underline{D}}$ -GlcNAc residue and asparagine with little or no degradation of the 2-deoxy-2-trifluoroacetamido-1-N-trifluoroacetyl- β - $\underline{\underline{D}}$ -glucopyranosylamine residue formed. This residue is converted into a 2-amino-2-deoxy- $\underline{\underline{D}}$ -glucitol residue by reduction with sodium borohydride. Chromium trioxide oxidation of the acetylated oligosaccharide alditol resulted in a product which on sugar analysis gave $\underline{\underline{D}}$ -Gal, $\underline{\underline{D}}$ -GlcNAc, $\underline{\underline{D}}$ -Man and $\underline{\underline{D}}$ -GlcNAc-OL in the molar proportions 0.3:0.0:2.1:1.0. This finding demonstrates that the branched $\underline{\underline{D}}$ -Man residue and the $\underline{\underline{D}}$ -GlcNAc unit substi-

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